

Ecological significance of amino acids and metal ions

A microanalytical approach

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ECOLOGICAL SIGNIFICANCE OF AMINO ACIDS AND METAL IONS

A MICROANALYTICAL APPROACH

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FK, Sm

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Abstract: Various aspects of population ecology were examined with amino acids and metal ions in different test systems, using micromethods of analytical chemistry. Amino acid derivatives were separated on capillary columns and determined by gas chromatography - mass spectrometry. Compensation of baseline drift during temperature programming was achieved by displacement of the electrometer voltage. Use of small reagent tubes and reagent amounts virtually eliminated background contamination in determination of metal ions in microsamples of biological material by atomic absorption spectrophotometry. The methods were used to study some mechanisms that determine fungal contribution to leaf decay, variations in the cost of exudation of amino acids from invertebrates, and the effects of metal ions on growth, population density and herbivore-plant relations of invertebrates and fungi. Fungi hydrolyze proteins in leaves and simultaneously absorb free amino acids in the surrounding water. A switch in behaviour for amino acid absorption was tested in an experiment, and fungi on alder leaves showed evidence for a superproportional absorption of free amino acids. Two species of fungi were isolated from leaves and it was demonstrated that differences in leaf chemistry were important for differences in habitat selection of the two species. The normal exudation of free amino acids by the isopod <i>Asellus aquaticus</i> was increased considerably in the presence of the amphipod <i>Gammarus pulex</i> . Concentrations of copper and lead ions in vital tissues such as cerebral ganglion and seminal vesicles in earthworms from Gusum in Östergötland, where a brass mill has contaminated the environment, were inversely proportional to the distance from the mill, while population density and biomass were proportional to the distance. A springtail species, <i>Onychiurus armatus</i> , which is common in the Gusum area, was found to take up metal ions to a steady state level, which was related to the metal ion concentration in its food. In an experiment, growth rate and body length were reduced by copper and lead concentrations that were similar to those found in specimens in the vicinity of the brass mill. A soil fungus was used to illustrate that periodic grazing by the springtail increased fungal respiration and growth, regardless of metal contamination of the fungal growth substrate.			
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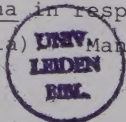
DISSERTATION

Lund 1982



The thesis is based on the following papers:

- I. Bengtsson, G. and Odham, G. 1979. A micromethod for the analysis of free amino acids by gas chromatography and its application to biological systems. - Anal. Biochem. 92:426-443.
- II. Bengtsson, G., Odham, G. and Westerdahl, G. 1981. Glass capillary gas chromatographic analysis of free amino acids in biological microenvironments using electron capture or selected ion-monitoring detection. - Anal. Biochem. 111:163-175.
- III. Bengtsson, G. and Cavallin, C. Compensation of baseline drift in temperature programmed capillary gas chromatography with electron capture detection. - J. Chromatogr. (in press).
- IV. Bengtsson, G. Switching for amino acids in aquatic hyphomycetes. - Manuscript.
- V. Bengtsson, G. Habitat selection in two species of aquatic hyphomycetes. - Manuscript.
- VI. Bengtsson, G. Energetic costs of amino acids in the interaction between the predator Gammarus pulex L. and the prey Asellus aquaticus L. - J. Chem. Ecol. (in press).
- VII. Bengtsson, G. and Gunnarsson, T. A micromethod for the determination of metals in biological tissues by furnace atomic absorption spectrophotometry. - Manuscript.
- VIII. Bengtsson, G., Gunnarsson, T. and Rundgren, S. Growth changes caused by metal uptake in a population of Onychiurus armatus (Tullb.) (Collembola) feeding on metal polluted fungi. - Oikos (in press).
- IX. Bengtsson, G., Nordström, S. and Rundgren, S. Population density and tissue metal concentration of lumbricids in forest soils near a brass mill. - Environ. Pollut. (in press).
- X. Bengtsson, G. and Rundgren, S. Respiration and growth of a fungus, Mortierella isabellina in response to grazing by Onychiurus armatus (Collembola) Manuscript.



Omgiven av så många goda rådgivare och kollaboratörer kan jag inte annat än beklaga att avhandlingen inte blev bättre. Det är, med en lätt travesti på ett gammalt syftningsfelexempel i real-skolan, en sammanfattning av hur jag uppfattat den process som fört fram till någon slags produkt av forskarutbildningen, i vid bemärkelse. Listan över dem som, medvetet eller omedvetet, påverkat min utbildning i allmänhet och pappersproduktionen i synnerhet ryms inte i denna skrift. Här skall blott några få i den omedelbara omgivningen ihågkommas.

Per Brinck och Göran Odham har varit mina handledare. Per, som alltid haft full förståelse för hur utrymneskrävande min verksamhet varit, har varit en drivande kraft bakom den upprustning av ekologisk kemi som varit en förutsättning för mitt arbete. Liksom Göran Odham har han slitit ner några rödpennor på att hitta fel och oklarheter i mina manuskript. Göran har lärt ut mycket kemi och apparatkunnande och ständigt varit på jakt efter manuskript, långt innan experimenten varit avslutade. Anders Södergren, som redan tidigt stjälpde ut sina försöksfiskar från ekokem för att jag skulle få någonstans att sitta, inspirerade mig att arbeta med miljögifter, och Sten Rundgren, som tyckte det blev trist att åka ut och gräva gropar ensam, blev ansvarig för en annan slagsida i mitt bibliotek. Torsten Gunnarsson har slavarbetat flera år och gjort det mesta av det markekologiska arbetet.

Gunilla Westerdahl har hållit ordning på mig, gjort kolonner och masspektra under stark tidspress, och Börje Svensson har alltid hållit något outhärligt gömt i någon låda åt mig. Ola Haraldsson och Axel Hagström har gett mig förtur många gånger då jag kommit traskande med halvtaskiga ritningar och små tidsmarginaler, och Sune Arlebo har alltid haft en bil, en rörmokare eller ett rabatthäfte i bakfickan när det som bäst behövts. Anne Odham, som blivit sömnlös flera nätter av mina klottriga manuskript, har skrivit rent avhandlingen tillsammans med Ylva Zachrisson. Steffi Douwes och Eva Karlsson har renritat figurerna och Gunilla Lindquist har tålmodigt klustrat och klippt under tidspress.

Några får jag tacka kollektivt. Ekokemiska avdelningen, som stått ut med mig under alla dessa år, avdelningen för mikrobiologisk ekologi, där jag alltid funnit någon med goda råd, studenter och lärarkollegor, som inspirerat mig till djupdykningar inom flera områden av ekologi, och kamraterna i ekologicirkeln, där vi ansträngt oss att syssla med sådant som ingen av oss vet särskilt mycket om.

INTRODUCTION

The thesis deals with the effects of two groups of chemicals - amino acids and metal ions - on some fungi and invertebrates in aquatic and terrestrial environments. The amino acids can be utilized as a nitrogen and energy source, whereas the metal ions may be stimulatory, inhibitory or toxic. Sampling difficulties have long limited studies of the effects of low, natural concentrations of amino acids and metal ions on fungi and invertebrates. To overcome this basic problem, micromethods for the analysis of amino acids and metal ions were developed. Such sophisticated methods of analytical chemistry open up new fields of interest in ecology. Similarly, ecological systems designed to test hypotheses in microcosms, require development of sensitive micromethods. At its best, a recurrent and profitable interaction.

Various aspects of population ecology were examined with amino acids and metal ions in different test systems. Switching behaviour and habitat selection directed by amino acids were shown for aquatic hyphomycetes, and variations in the costs of exudation of amino acids were estimated from the interaction between Gammarus pulex and Asellus aquaticus. The effects of metals on growth, population density and herbivore-plant relations were studied on soil invertebrates and fungi.

SUMMARY OF RESULTS

The need for a simple, rapid and sensitive method to determine small amounts of amino acids resulted in papers I-III. At the time when the choice of the analytical technique was made, gas chromatography offered certain advantages, such as the supply of equipment and the technical experience of the laboratory staff. Due to low volatility amino acids have to be derivatized for GC-analysis. It was done by a combination of esterification and acylation to a N-heptafluorobutyryl isobutyl ester derivative (paper I). Sample treatment included a clean-up step with chloroform extraction and cation exchange. Evaporation losses were reduced to a minimum by use of capillary tubes, and the samples could be concentrated without significant losses from 1000 ml to 5 µl prior to analysis. Nineteen amino acids were separated on a packed glass column, a WCOT column and a SCOT column, which were all prepared in the laboratory. The method was illustrated by

analyses of amino acids in lake water and stream water, and exudation from the nematophagous fungi Arthrobotrys oligospora. With the mass spectrometer used as a detector, the detection limit was reduced 1000 times compared with flame ionization detection, and a few picograms of selected amino acids were determined in a complex amino acid mixture.

The efforts to increase the sensitivity were extended in paper II, and detection limits for amino acids at less than 1 pg were found. Electron capture detection and selected ion monitoring were compared and applications of the method demonstrated on samples collected from the surface microlayer and subsurface water of a lake, honeydew from aphids, dew of the Lady's mantle, nematode and mycoplasma exudates. Bleeding from the stationary column was a major problem when small quantities of amino acids were determined by electron capture detection. The compensation of the severe baseline drift was achieved by a simple displacement of the electrometer voltage (paper III).

Papers IV-VI are based on the method developed in papers I-III. Papers IV and V are concerned with some mechanisms that determine fungal contribution to leaf decay. Fungi occur on all sorts of decaying leaves, more numerous on some leaves than on others. Eleven species were found on alder leaves and three species on beech leaves. The fungi grew well when the leaves were incubated in the laboratory, and used 55-75 % of the amount of amino acids in the leaves during 9-10 weeks of incubation. Simultaneously, free amino acids in the water surrounding the leaves were absorbed by the fungi, more rapidly and efficiently by fungi on alder leaves. Grazing of the fungi by the amphipod Gammarus pulex reduced the free amino acid absorption but maintained the growth of the mycelium. A switching in amino acid absorption was tested for fungi on alder leaves. When less than 30 % of the available amino acids were free and dissolved in the water, even less were absorbed, whereas absorption became superproportional when more than 40 % of the available amino acids were dissolved. Concentrations of dissolved amino acids similar to those required for switching were found in leaf drifts in natural stream water.

In order to explain the differences in amino acid absorption between fungi on alder and beech leaves, two species were isolated and inoculated on sterilized leaves (paper V). Tetracladium marchalianum was abundant on alder leaves but absent on beech leaves, whereas Tricladium splendens was absent on alder leaves

and dominating on beech leaves. Several parameters were determined in an experiment, viz. leaf weight loss, mycelial length, protease activity and concentration of leaf protein, dissolved amino acids, ammonium and nitrate. It was shown that differences in leaf chemistry could not be rejected as an explanation for differences in abundance of the fungal species on the leaves.

Compared with leaf leachate, the amounts of amino acids exuded from small organisms in water are negligible and would elude detection in a natural water. The exudation loss can be estimated in experiments, as demonstrated in paper VI, and the significance of exudation illustrated. The isopod Asellus aquaticus displayed avoidance behaviour in exudates from the amphipod Gammarus pulex and increased its normal exudation of amino acids considerably. Asellus lost 4-5 % of its assimilated energy as amino acid exudates when Gammarus was present in its neighbourhood.

The weight of a patch of fungal mycelium, of a microarthropod or the cerebral ganglion of an earthworm is only some tens of a microgram. Determinations of metal ions in tissues of such low weight is not a straightforward procedure and several approaches, including concentration steps by solvent extractions, have been suggested. Paper VII demonstrates a micromethod for metal ion determination, which virtually eliminates background contamination by use of small reagent tubes and reagent amounts. The precision of the method was good, and it could be shown that there was considerable variation in the metal contents of biological material from the same environment. This is evident from the data on copper and lead in tissues of earthworms from Gusum in Östergötland, where a brass mill has contaminated the environment (paper IX). Copper concentrations were higher than lead concentrations both in soil and earthworm tissues and were inversely proportional to the distance from the mill. Higher concentrations were found in juvenile specimens compared to adults, and increased metal levels were common in the seminal vesicles and the cerebral ganglion of earthworms close to the mill, which may have some importance for the survival of local populations. Population density and biomass were proportional to the distance from the mill, which indicated the effects of metal pollution on survival of earthworms.

The micromethod developed in paper VII was used to monitor the uptake and the release of copper and lead in a springtail species, Onychiurus armatus, which was reared on a soil borne fungus, Verticillium bulbillosum (paper VIII). The fungus accumulated the

metal ions efficiently, and the springtails initially concentrated metal ions and then excreted towards a steady state level, which was related to the metal ion concentration in the fungal growth medium. Moderate contamination of the fungus increased the growth rate and average maximum length of springtails in both F1 and F2 generations, whereas contamination to the same level as in the vicinity of the brass mill at Gusum reduced growth rate and body length, especially in the F2 generation. Another soil fungus, Mortierella isabellina, was used to illustrate that metal ions in the growth medium increased the respiration of the fungus, which affected the growth rate of the mycelium (paper X). Periodic grazing by the springtail increased fungal respiration and growth, regardless of metal contamination, but the stimulation of the fungal growth in a metal polluted soil was slightly less than in an unpolluted soil.

A Micromethod for the Analysis of Free Amino Acids by Gas Chromatography and its Application to Biological Systems

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A gas chromatographic procedure was developed for determination of minute amounts of free amino acids in natural waters and laboratory models simulating biological systems. Sample pretreatment included removal of interfering organic substances by chloroform extraction and isolation of amino acids by cation exchange. Amino acids were converted to their *N*-heptafluorobutyl isobutyl ester derivatives in glass capillary tubes, permitting considerable concentration of the sample prior to gc injection. The derivatives of 19 amino acids were successfully separated on either a glass column packed with a mixture of OV-101 and OV-17 on Chromosorb W, a glass capillary column coated with OV-101, or a support-coated capillary column supported with SE-30. One to five nanograms of individual amino acids were detected using flame ionization detector. The detection limit was reduced more than 100-fold using the electron capture detector and more than 1000-fold by mass fragmentography. The procedure allowed determination of less than 1 ppb of individual amino acids in lake and river water samples and was used to estimate the excretion of free amino acids from microbial populations.

The advantages of gas chromatography in comparison with ion-exchange chromatography for the analysis of amino acids with respect to sensitivity, operating time and costs are probably the main reasons for the increased rate of publications in the 1970s of improvements and modifications of gas chromatographic (gc) techniques (1-19). The purpose of several of these contributions was to obtain complete separation of individual components on a single column, reliable quantification down to the picomole level, and rapid identification of unknown amino acids by combined gc-mass spectrometry.

One of the most attractive volatile amino acid derivatives is the *N*-heptafluorobutyl (HFB)¹ alkyl ester (10-13,15,17,18). The HFB anhydride forms relatively stable

amino acid derivatives that are suitably volatile for practical handling and allow reasonably short analysis time on the gc. In addition, the fluoroderivatives give a very high response with electron capture detection (18,20,21). A significant improvement in separation, which is desirable when analyzing amino acid enantiomers and complex biological samples, has been obtained by the use of glass capillary columns (2,7). When these are connected to the mass spectrometer, excellent sensitivity for detection of individual amino acids using the selected ion monitoring technique can be attained.

Comparatively little information is available about free amino acids in natural systems. Although only small amounts occur in natural waters—from less than 1 to 20 $\mu\text{g/liter}$ of individual amino acids (22-26)—much evidence supports the hypothesis that

¹ Abbreviations used: HFB, *N*-heptafluorobutyl; SCOT, support-coated HFBA, heptafluorobutyric anhydride; BHT, 2,5-di-*tert*-butylhydroxytoluene; D2, distilled and deionized; PVC, polyvinyl chloride;

WCOT, wall coated; DMCS, dimethyldichlorosilan; ECD, electron capture detection; NPD, nitrogen-phosphorus detection.

the free amino acid pool constitutes an energy and nutrient source for microorganisms, phytoplankton, and invertebrates (27), although its relative contribution to, e.g., the energy budget, of these groups is hardly known. Probably microbial uptake is predominant (27). As a source of reduced nitrogen, the amino acids might support phytoplankton growth (28,29). Uptake and excretion of amino acids have been demonstrated for invertebrates, in sea water [c.f. review in (30)]. It still remains to assess the advantage of amino acids for invertebrates; it is argued that the growth and reproductive rate of an individual may be increased, producing more suitable conditions for natural selection.

Most works dealing with gas chromatographic determinations of amino acids involve proteinaceous starting materials. Usually, techniques allowing estimations down to the microgram level have been used. The purpose of the present study is to develop a simple and reliable method for separating amino acids on a single column and for analysis of free amino acids occurring in natural waters in the nanogram range. Gehrke *et al.* (31) detailed methods with a similar aim in connection with the lunar investigations. The present method is designed so that it can also be applied to experimental studies of the transport, distribution, and metabolism of free amino acids in laboratory models at natural concentrations.

MATERIALS AND METHODS

Gas Chromatography

Perkin-Elmer Models 900 and 3920 gas chromatographs, equipped with dual-flame ionization detectors and a linear temperature programmer, were used. Samples were analyzed on a 3 m $\times$ 2 mm i.d. glass column packed with a mixture of OV-101 and OV-17 (2.5:0.8% or 2.0:2.0%) on Chromosorb W, HP, 80-100 mesh. Prior to packing, the column was cleaned with 5% Deconex 11 (Borer Chemie, Switzerland) for 24 h, HCl (37%) for 1 h, and treated with 2% DMCS

in toluene for 2 h. The chromatographic conditions were: injector temperature, 250°C; detector temperature, 290°C. The oven was initially held isothermal at 85°C for 7 min, then linearly programmed at a rate of 2.5 or 4°C/min to 230°C. The nitrogen-carrier gas flow was 30 ml/min.

A Perkin-Elmer Model 3920 gas chromatograph modified for glass capillary columns was employed. Samples were detected with a flame ionization detector.

Usually, a 25-m glass capillary column (i.d. 0.3 mm) coated with OV-101 according to (32) with an all-glass inlet-splitting device was used. This was constructed in the laboratory to give a minimum dead volume and was equipped with a small space (0.5 ml) filled with silanized glass beads (20-40 mesh) just before the splitting point to ensure linear splitting. The glass body was attached to a needle valve via a buffer volume (1.5 ml) allowing continuous adjustment of the split ratio. The split ratio was held at 10:1. The average liquid-phase film thickness was calculated as 0.3 μ m. The chromatographic conditions were: injector temperature, 250°C, detector temperature, 290°C. The column temperature was held at 85°C for 4 min, then programmed to 215°C at 8°C/min. The carrier gas (nitrogen or helium) flow rate was 1.9 ml/min.

Alternatively, a 24-m glass SCOT capillary column (i.d. 0.5 mm), supplied with silanized and deactivated SE-30 (Scientific Glass Engineering, Australia), was used. It was installed with an all-glass splitless injection system. The chromatographic conditions were: injector temperature, 230°C; detector temperature, 290°C. The temperature program was 85°C for 4 min, then programmed at a rate of 4°C/min to 200°C. Nitrogen-carrier gas flow was 5 ml/min.

A Varian Aerograph Model 200, equipped with a ^3H electron-capture detector (33), was used for a study of detection limits. Samples were analyzed on a 2 m $\times$ 1.5 mm i.d. glass column packed with a mixture of 4% SF-96 and 8% QF-1 on Chromosorb W,

HP, 80–100 mesh. The chromatographic conditions were: injector temperature, 205°C; detector temperature, 220°C. The oven temperature was held at 150°C for 20 min, then programmed to 200°C at a rate of 8°C/min. Nitrogen-carrier gas flow was 30 ml/min.

A Varian MAT-112 gc-ms system was used for the identification of structure and mass fragmentation. The gas chromatograph was equipped with a glass capillary column coated with OV-101 as described. The ion source was held at 250°C and electron energy was 75 eV.

Reagents

Analytical grade amino acids were obtained from Fluka Chemie, Switzerland. Stock solutions of each amino acid were prepared in 0.1 M HCl at concentrations of 0.25 mmol/ml and stored in screw-cap culture tubes at 4°C.

Dowex 50W (H⁺), 100–200 mesh, and Amberlite IR-120 (H⁺), 28–35 mesh, were obtained from Fluka. Before storing, the resins were cleaned twice by alternately washing in 3 M HCl and 3 M NH₄OH.

Concentrated HCl, NH₄OH, *n*-butanol, isobutanol, chloroform, dichloromethane, acetylchloride, ethyl acetate, and acetic anhydride were of analytical grade and obtained from Merck. They were not further purified. Heptafluorobutyric anhydride (HFBA) and 2,5-di-*tert*-butylhydroxy-toluene (BHT) were from Fluka.

Preparation of Ion-Exchange Column

Dowex 50W (H⁺) (100–200 mg) was placed in a Pasteur pipet with a glass wool plug in the stem. Distilled and deionized (D2) water was used to wash the resin and fill the pipet, which was connected to a glass funnel by a short piece of PVC tubing. Several pipets were arranged on a stand.

The resin was loaded with 4 ml of 3 M HCl and then washed with (D2) water until the eluate was neutral. After elution of the

sample, the resin was washed with 15 ml of (D2) water to pH 5 to 6 and then regenerated with 4 ml of 3 M HCl. Each resin was used three times.

With the Amberlite IR-120 (H⁺), 50 cm³ was placed in a glass column (300 × 2 mm i.d.) fitted with a sintered disk. The column was loaded with 50 ml of 3 M HCl, washed with (D2) water, and, after elution, regenerated with 50 ml of 3 M HCl.

Aqueous Sample Pretreatment

Sample volumes ranging from 1000 (field samples) to 3 to 500 ml (laboratory experiments) were filtered through a membrane filter (Millipore, mixed cellulose acetate and nitrate, pore size 0.45 μm) to remove particulate matter. The filtrate was further treated in one of two ways.

Method A. The sample was evaporated in a rotary evaporator at 80°C and reduced pressure. The initial volume was reduced to 2 to 10 ml, depending upon the amount of salts and organic matter (in respect to specific conductivity the treated samples represented 50–350 μS), and transferred to a 50-ml test tube. The evaporator flask was washed with 3 × 2 ml of (D2) water which was added to the sample.

HCl (3 M) was added to the tube to give a pH of 2.5 to 3.0, followed by 2 ml of chloroform (previously equilibrated with water to remove ethanol). The sample was vigorously shaken for 10 min. The tube was then centrifuged for 5 min and the aqueous phase transferred to another tube. Water [2 × 1 ml (D2)] was carefully added to the chloroform surface, sucked off, and added to the sample. The combined aqueous phases were then extracted with another portion of 2 ml of chloroform and transferred to the glass funnel attached to the Dowex 50W resin.

The sample was allowed to pass through the resin at a rate of about 1.0 ml/min. The system was washed with 5 ml of (D2) water to remove neutral molecules and anions from the column. Then 3 ml of 3 M NH₄OH

was added to the funnel and the amino acids were eluted. The eluate (3.5 ml) was collected in a Teflon-lined screw-cap culture tube, 14 × 90 mm, and evaporated until almost dry in a rotary evaporator at 70°C. The tube was connected to the evaporator by a threaded Teflon cap.

Method B. The sample was allowed to pass through the Amberlite column at a rate of 16 ml/min. The funnel was washed twice with 25 ml of (D2) water. The amino acids were eluted with 150 ml of 3 M NH_4OH . The eluate was collected in a 250-ml round-bottomed flask and reduced to 5 to 10 ml in an evaporator. Further treatment included chloroform extraction as described previously, and the sample was subsequently evaporated until almost dry.

After pretreatment by method A or B, the aqueous sample was sucked up with a pipet-filler (Pumpett 18 mikro, Sweden) in a Pyrex glass capillary tube, 80 × 2 mm i.d. The culture tube was washed twice with 50- μl portions of (D2) water, each of which was sucked up the capillary tube and added to the sample.

The aqueous column was sucked up the tube a distance of approximately 1.5 cm to the end. With the pipet-filler still attached, the capillary tube was sealed in a micro-flame. By gently tapping the tube, the contents fell to the base of the sealed capillary. The pipet-filler was then removed and the sample was evaporated to dryness overnight in a desiccator (10–20 mm).

Derivatization

Twenty-five microliters of dichloromethane was added to the glass tube and traces of water were removed azeotropically under vacuum in the desiccator. Fifty microliters of *n*- or isobutanol–3 M HCl was added to the glass tube, which was flushed with nitrogen and sealed. The esterification reagent was prepared by adding the appropriate amount (5:20 v/v) of acetyl chloride to the alcohol.

After stirring in an ultrasonic bath for 5 min, the tube was placed in an oil bath at 120°C for 20 min. The tube was immediately cooled in an ice bath. It was then opened and the excess reagent was evaporated in a desiccator at reduced pressure (approximately 3 h using an aspirator pump followed by 30 min using a vacuum pump). Twenty-five microliters of dichloromethane was added and evaporated followed by 25 μl of BHT (approximately 10^{-6} M), dissolved in ethyl acetate, and 25 μl of HFBA. The tube was flushed with nitrogen, sealed, and then heated in an oil bath at 150°C for 10 min. The tube was immediately cooled in an ice bath, opened, and the excess reagent was evaporated in a desiccator at reduced pressure (c.f. above).

The sample was dissolved in 5 μl of anhydrous ethyl acetate and stored in a sealed tube at –20°C until analyzed. At the time of injection 0.5 μl of acetic anhydride was coinjected with 1.0 μl of sample to obtain an on-column conversion of the monoacyl to the diacyl derivative of histidine (c.f. 7,15,34). A schematic diagram of the pretreatment and derivatization procedure is given in Scheme 1.

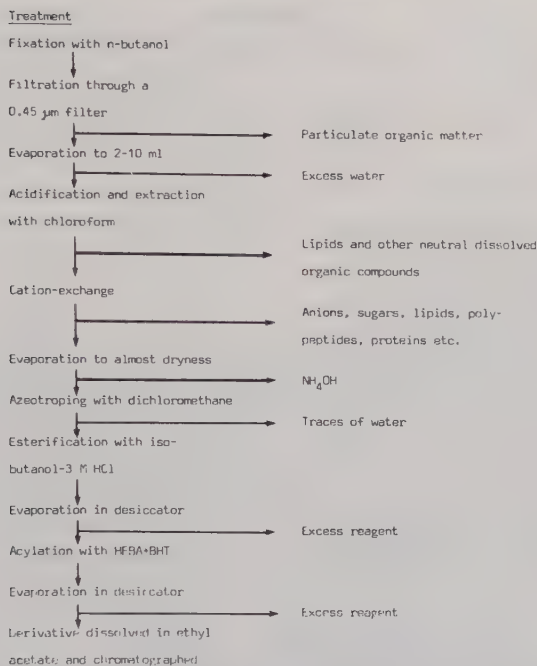
RESULTS

Choice of Reaction Tube for Derivatization

Five identical standard samples (containing 20 amino acids of approximately 100 ng each) were run with the capillary glass tube and five with the culture tube. Peak areas were measured for each amino acid and their sum in each sample calculated. The difference between the means of the peak area sums of capillary tube and culture tube tests was less than 0.5%, which is the same order of magnitude as the SD within each category.

Losses during Pretreatment

Four 1000-ml standard mixtures (containing approximately 100 ng each of 19 amino



SCHEME 1. Schematic diagram of the pretreatment and derivatization procedure for gc analysis of amino acids.

acids) were run through the evaporation procedure. The losses of amino acids were calculated by comparison with nonevaporated samples of the same origin. Losses occurred, although too small to be significant ($P = 0.32$), (c.f. Table 1).

No systematic losses of amino acids from the aqueous phase were found during the chloroform extraction procedure, provided ethanol had previously been removed from the chloroform.

With respect to the ion exchange procedure the composition of resin, pH, and the amount of resin substance were investigated (Fig. 1). It was found that direct desalting of 100 ng of amino acids on the Amberlite

IR-120 gave unreliable results. In several cases, a small residue of a viscous liquid was found in the capillary tubes after evaporation. Subsequent derivatization yielded less than 50% of amino acids, independent of the pH of the sample.

The yield using Dowex 50 was acceptable (Table 1). Clearly, the performance of the ion-exchange procedure is highly dependent on the pH of the sample. The amount of resin (50–200 mg) was found to be non-critical.

Derivatization

The optimal reaction time for the simultaneous esterification of all amino acids was

TABLE I

PRETREATMENT RECOVERIES AND EFFECT ON DERIVATIZATION OF THE CHOICE OF ESTERIFICATION REAGENT^a

		Derivatization with isobutanol-3 M HCl	Derivatization with <i>n</i> -butanol-3 M HCl
Nonevaporated sample	$\bar{X}$	99.86 (A)	98.15 (B)
	<i>s</i>	0.17	1.80
Evaporation of a 1000-ml sample	$\bar{X}$	99.84 (C)	
	<i>s</i>	0.17	
Evaporation of a 1000-ml sample and cation exchange with 100 mg of Dowex 50, pH 4.5	$\bar{X}$	36.27 (D)	
	<i>s</i>	33.35	
Evaporation of a 1000-ml sample and cation exchange with 100 mg of Dowex 50, pH 2.5 to 3.0	$\bar{X}$	99.77 (E)	
	<i>s</i>	0.16	

^a Derivatization was performed in glass capillary tubes. For acylation, equal amounts (v/v) of HFBA and BHT dissolved in ethyl acetate were used. Approximately 100 ng of a mixture of amino acids was injected on a glass column supported with OV-101. Peak areas were calculated for individual amino acids in percentage of the largest area in all runs and the mean percentage recovery for all amino acids calculated for each combination of parameters included in the table. A *t* test was made with the following results: A/B: *t* = 5.81, *P* < 0.001; A/C: *t* = 0.45, *P* = 0.324; A/D: *t* = 11.75, *P* < 0.001; A/E: *t* = 2.22, *P* = 0.014. $\bar{X}$, mean percentage area in a run; *s*, standard deviation; *n*, 38.

found to be 20 min at 120°C (Table 2). The optimal proportion of isobutanol and acetyl chloride was 20:5, corresponding to about 3 M hydrochloric acid. The yield decreased

2% at a concentration of 1.5 M HCl and 7% at 5 M. The most volatile amino acid derivatives accounted for the major part of the observed decrease at the low concentration

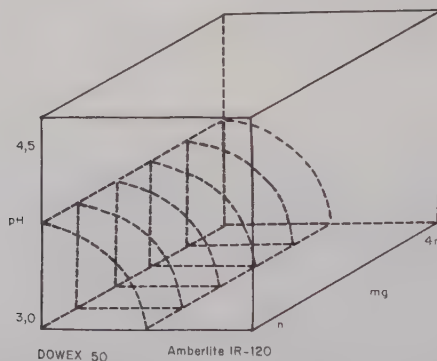


FIG. 1. An impressionistic illustration of the optimal conditions of the ion-exchange procedure. Variation of the three parameters, pH, particle surface area, and resin amount (e.g., *n* = 50 for Dowex 50), within the marked volume does not influence the yield. An increase of pH to 4.5 reduces the yield drastically, while it is insignificantly affected, at least when using a resin with a large particle surface area, by acidification of the sample to less than pH 3.5. Biological samples were found easier to handle at pH 2.5 to 3.0 than lower. Although the yield is also optimal at lower pHs these were omitted for practical reasons. Reducing the particle surface area implies a restriction of the optimal pH interval.

of hydrochloric acid. It was found that by substituting *n*-butanol for isobutanol, a significant increase (Table 1) in yield was obtained.

Acylation should be performed at 150°C (Table 3). The acylation time is less critical. However, the sample means of 150°C/10 min and 150°C/15 min are significantly different at the 5% level. Optimal time for a complex amino acid-mixture analysis was 10 to 12 min, although the yield of the most high-boiling derivatives was not maximal.

Separation Technique

The primary goal of the gc separation was to achieve sufficient separation of the common amino acids on a packed glass column at the level of 1 to 10 ng. The first column investigated was packed with 2.5% SE-30 on Chromosorb W, HP, and gave satisfactory separation of about two-thirds of the common amino acids. A second column was

TABLE 2
ESTERIFICATION EXPERIMENTS^a

Temperature (°C)		Time (min)		
		10	20	30
110	$\bar{X}$			98.56
	<i>s</i>			1.04
120	$\bar{X}$	97.97	99.73 (A)	99.12 (B)
	<i>s</i>	1.91	0.31	0.67
130	$\bar{X}$	98.77	98.23	
	<i>s</i>	0.83	1.23	

^a Esterification was done in glass capillary tubes with 50 μ l of isobutanol-3 M HCl followed by acylation with HFBA + BHT in ethyl acetate. Each sample, consisting of approximately 100 ng of each amino acid, was injected on a 3 m $\times$ 0.2 mm i.d. packed column with 2.5% OV-101 and 0.8% OV-17. Peak areas were calculated for individual amino acids in percentage of the largest area in all runs and the mean percentage recovery for all amino acids calculated for each combination of parameters included in the table. *Z* test (*n* = 57) was made at A and B, *z* = 6.24, *P* < 0.001; as well as *F* test, *F* = 4.67, *P* < 0.01.

TABLE 3
ACYLATION EXPERIMENTS^a

Temperature (°C)		Time (min)		
		10	15	20
100	$\bar{X}$			98.97
	<i>s</i>			0.88
130	$\bar{X}$			99.40 (C)
	<i>s</i>			0.58
150	$\bar{X}$	99.85 (A)	99.69 (B)	
	<i>s</i>	0.17	0.39	

^a Esterification was made in glass capillary tubes with isobutanol-3 M HCl at 120°C/20 min and acylation with HFBA + BHT in ethyl acetate. The injection was made of approximately 100 ng of each amino acid. Gas chromatographic conditions were as in Table 2, and calculations were made in the same way. Result of *t* tests (*n* = 38): A/B, *t* = 2.35, *P* = 0.021; A/C, *t* = 4.60, *P* = 0.00001.

filled with 2.5% OV-101 on the same adsorbate and the separation efficiency was significantly enhanced, although difficulties in separating aspartic acid and phenylalanine still remained. Since the two stationary phases are practically identical with respect to the McReynolds constants, it is possible that the observed differences in separation efficiency are due to individual differences of the columns. In an attempt to obtain better resolution, the adsorbent was coated with a mixed, slightly more polar stationary phase consisting of 2.5% OV-101 (methyl silicone) and 0.8% OV-17 (50% methyl silicone, 50% phenylsilicone). The improvement was, however, not obvious until isobutanol was used for esterification. Furthermore, only when acetic anhydride was coinjected with the sample was a sharp peak found for histidine. Additional progress was made when the proportion of phenylsilicone was increased (2% OV-101 + 2% OV-17). Twenty amino acids were resolved in 50 min in a single run on the column using a temperature-programming rate of 2.5°C/min (Fig. 2).

In order to further improve the separa-

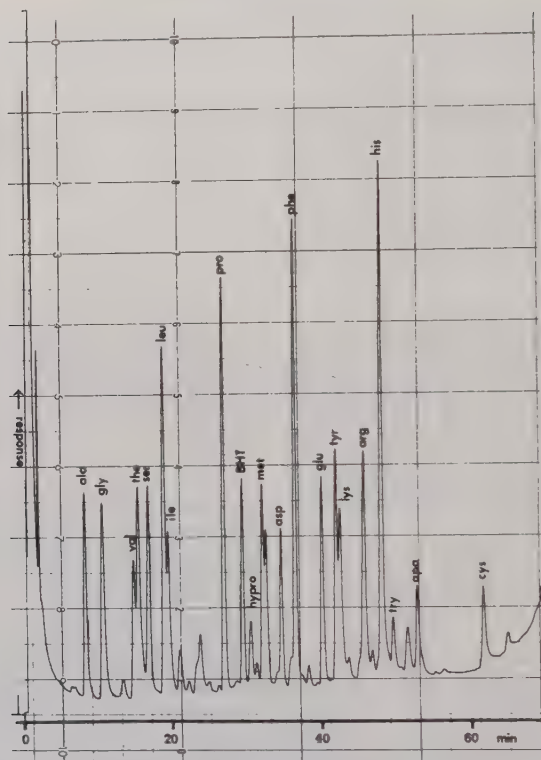


FIG. 2. Glc of a mixture of 20 amino acids on a 3 m $\times$ 2 mm i.d. glass column packed with a mixture of 2% OV-101 and 2% OV-17 on Chromosorb W, HP, 80–100 mesh. Initial temperature, 85°C; programming rate, 2.5°C/min; and final temperature, 235°C. The amino acid peaks represent 100 to 150 ng. Aminopimelic acid was used as the internal standard. The unlabeled peaks are mainly due to impurities of the amino acid standard.

tion, glass capillary columns were tested. It was found that in addition to better separation, the 25-m capillary column coated with OV-101 allowed more rapid analysis—a complete chromatogram was obtained in 35 min. Probably due to inefficient operation of the inlet split system, the most high-boiling derivatives were difficult to reproduce quantitatively. Although the separation

power of the SCOT column was not as good as that of the wall-coated (WCOT) glass capillary column, it exceeded that of the packed columns. As a splitless injection technique can be used, the SCOT column can be operated with low concentrations of amino acids. Figure 3 shows the separation of amino acids on the SCOT column. Independent of column systems, helium-carrier

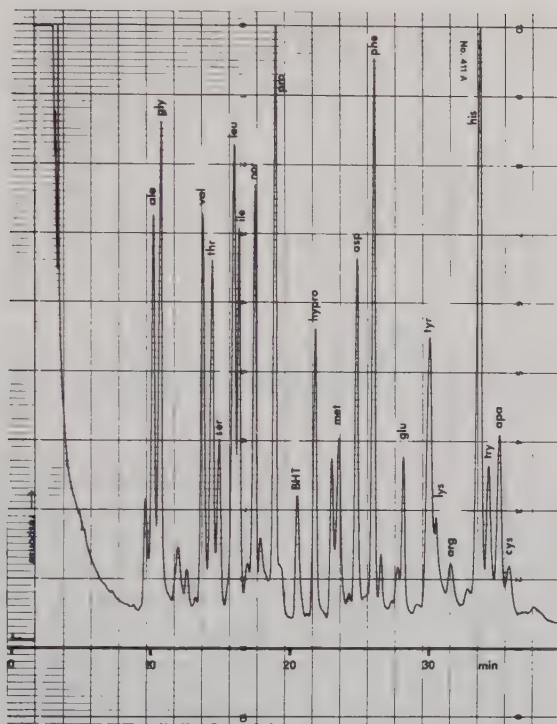


FIG. 3. Glc of a mixture of amino acids on a 24-m glass SCOT column supported with SE-30. Initial temperature, 85°C; programming rate, 4°C/min; and final temperature 210°C. The peaks represent 100 to 200 ng.

gas was found superior to nitrogen with respect to resolution.

Detection Technique

The linearity of the flame ionization detector over the range of 2 to 100 ng was studied on a packed column for some representative amino acids including serine, leucine, and methionine (Fig. 4). The detection limit for most amino acids was found to be about 1 to 5 ng using this detector. With an

electron capture detector, less than 10 pg of several amino acids could be detected.

Using the mass spectrometer as detector, 0.8 pg of valine could be detected without difficulty monitoring at the fragments of *m/e* 268 (35). Due to problems in avoiding contamination from naturally occurring valine in the laboratory environment, the detector signal did not decrease at lower amounts. It can, however, be concluded that this method has the capacity to detect subpicogram levels of amino acids.

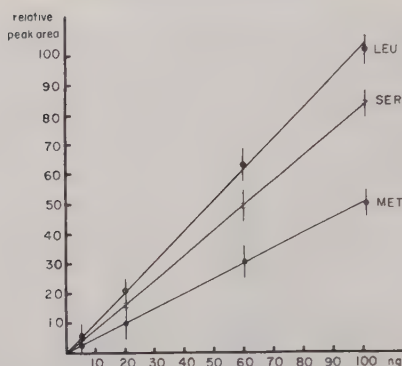


FIG. 4. Gas chromatographic peak areas as function of injected amino acid derivatives. The bars show the 95% confidence limits.

Application

As the main purpose of this work is to develop a gc method applicable to the study of microamounts of free amino acids in aquatic environments, some typical examples are demonstrated in Fig. 5 through 10. Lake Vombsjön (Fig. 5), eutrophic, and Lake Bosarpasjön (Fig. 6), mesotrophic, are situated in southern Sweden. Except for histidine and tryptophane, the highest concentrations of individual amino acids were found in the less productive lake, which also had a wider spectrum of amino acids. Less than half of the gc peaks were identified as common protein amino acids; the remaining, several of which are quantitatively important, have not yet been identified.

Stampenbäcken (Fig. 7) is a fairly small and unpolluted watercourse in southern Sweden (36). With two exceptions, the concentrations of individual amino acids are less than 1 ppb.

There are several sources of amino acids in a running water system. One of the most important is dead leaves from trees surrounding the watercourse. The excretion

of individual amino acids after 2 weeks from three medium-sized beech leaves from Stampenbäcken is in the range 3 to 8 ng/h (calculated as constant excretion which is probably not true) (Fig. 8). The excretion rate of amino acids from a laboratory population of the nematophagous fungi *Arthrobotrys oligospora* (Fig. 9) is on the order of 0.1 to 4 $\mu\text{g/h}$. This demonstrates the possibility of quantifying the excretion of amino acids from small populations of microorganisms in laboratory experiments. However, the method is evidently not limited to situations in which a fairly simple amino acid mixture occurs, e.g., in connection with experimental laboratory models, but can be used to determine the composition of complex amino acid mixtures surrounding organisms in natural aquatic environments.

Figure 10 illustrates an application of mass fragmentography for detection. An aqueous sample was treated as described and analyzed on the gc-ms system using a glass capillary column for separation. Monitoring at the peak of m/z 268, characteristic of valine, enabled easy identification and detection.

DISCUSSION

Any attempt to improve the gc method for amino acid analysis must by necessity deal with the problem of finding an optimal combination of the parameters speed, efficiency, reproducibility, and sensitivity. Usually the most time-consuming step is the pretreatment of the samples, in particular, the evaporation of various amounts of water. If the sensitivity can be increased, e.g., by concentration of the derivative, injection of a larger volume, and/or improvement of the detector, the original sample volume can be reduced.

An important improvement with respect to sample size concerns the use of capillary tubes for derivatization. The risk of evaporation losses (c.f. 7,10,18) when the deriv-

atives are concentrated to very small volumes is minimized. Furthermore, evaporation of submicrogram amounts of amino acid derivatives at room temperature at reduced pressure in a desiccator probably prevents hydrolysis of the derivatives by atmospheric moisture. Part of the success may be ascribed to the quality of the HFB derivatives, which have low volatility at room temperature and are reasonably stable with respect to hydrolysis (10,37). As has been mentioned, concentration of samples eluted from the Amberlite resin resulted in residues with an oily appearance when the volume reached about 15 to 50 μ l, and further reduction became impossible. This has been suggested as a possible limitation of the concentration procedure (18). However, it was found that the Dowex 50 resin

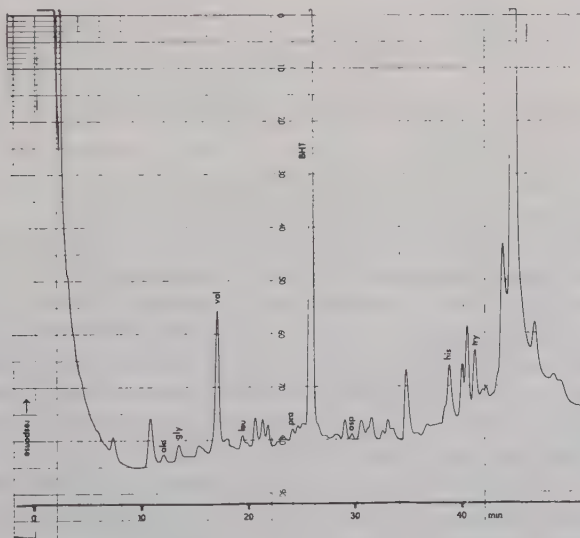


Fig. 5. Glc of amino acids in a surface-water sample of Lake Vombsjön. The amino acids were separated on a 3 m $\times$ 2 mm i.d. glass column packed with a mixture of 2.5% OV-101 and 0.8% OV-17. Chromatographic conditions are as in Fig. 2. Programming rate, 4°C/min. The amino acid peaks represent 10 to 300 ng. Remaining peaks are not identified.

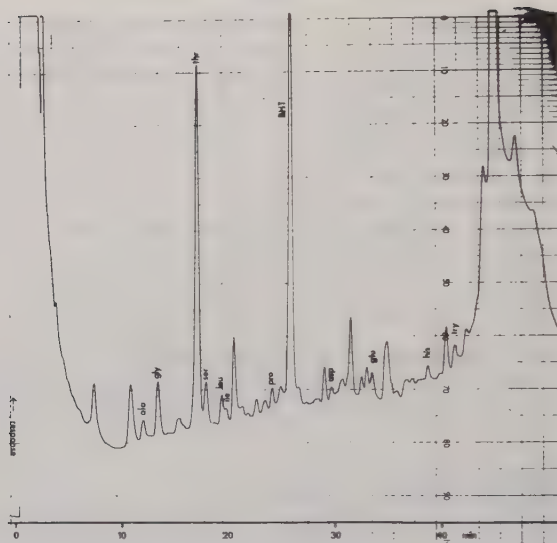


FIG. 6. Glc of amino acids in a surface water sample of Lake Bosarpassjön. The amino acid peaks represent 20 to 800 ng. Conditions are as in Fig. 5.

allowed the desired reduction and derivatization of the sample in a glass capillary tube and hereby increased the sensitivity of the gc method 10- to 20-fold. Furthermore, several samples can be processed simultaneously, and the costs of expensive reagents are reduced.

Although losses of amino acids during evaporation prior to derivatization are negligible, the very complex chemical composition of biological samples can be expected to affect a successful evaporation. Using a radioactive amino acid tracer, the magnitude of coprecipitation of small amounts of amino acids with carbonates and hydroxides might be estimated. However, addition of an amino acid standard to biological samples did not demonstrate any obvious loss. By acidifying the sample before evaporation to prevent, e.g., CaCO_3

precipitation, losses from nutrient rich samples should be further reduced.

When the original method was tested on a water sample from a productive lake, direct cation exchange proved to be inadequate in obtaining a sufficiently pure solution for derivative formation. It was found that extraction of the aqueous sample with chloroform prior to ion exchange efficiently removed interfering organic substances without detectable losses of amino acids. A similar observation has been made on eutrophic-lake water samples with ligand exchange and cation exchange combined for sample cleanup (38). These authors failed, however, to isolate glycine, glutamic acid and aspartic acid.

The procedures described in this paper are fairly simple. The only step that is not straightforward is the preparation of the

ion-exchange column, but this can easily be standardized. The resin amount is not critical within the range 50 to 200 mg and minor variations of the glass-wool packing only affect the flow rate.

The pH of the sample is one of the main factors regulating the displacement reactions on the sulfonic acid bed. In acid solution the amino groups are at least partly protonated whereas the ionization of the carboxyl group is very small. If an amino acid with a net charge of zero is passed through the resin, it is adsorbed to some extent because of the acid nature of the resin surface which brings the amino acid partly into the cationic form. Strong acidification is therefore necessary to bring the monoamino acids completely into the univalent cation form. For example, at pH 2.5, 35% of phenylalanine, 66% of threonine, and 100% of the diamino acids are in the cationic form. As many authors have remarked (see, e.g., 39) molecular adsorption due to electrostatic and van der Waals effects is very large and increases with molecular weight of the amino acid. The aromatic amino acids are in particular firmly held by the resin. The strong adsorption affinity of amino acids may well be partly responsible for the good recovery from the ion-exchange step. Further acidification of the resin did not affect the yield.

A similar cation-exchange technique has been used (1,2) and a recovery better than 94% of the individual amino acids, arginine excluded, was obtained. Cancalon and Klingman (4) lost up to 50% of methionine, phenylalanine, and tyrosine and 100% of tryptophane in a two-step ion-exchange procedure, including cation exchange on 200–400 mesh Dowex 50 $\times$ 8 and anion-exchange on 200–400 mesh Dowex 1 $\times$ 8. Their findings agree with those of Krutz (40) who recovered only minute amounts of cystine, methionine, and tyrosine, and phenylalanine after ion exchange on 20–50 mesh Amberlite IR-120 and 20–50 mesh Amberlite IRA-400. For quantitative work,

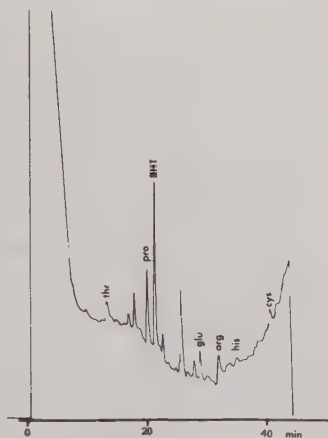


FIG. 7. GLC of amino acids in a water sample from Stampenbäcken. The amino acid peaks represent 1 to 20 ng. Chromatographic conditions are as in Fig. 2. Programming rate, 4°C/min.

the anion-exchange procedure should be further developed.

It may be suspected that the sulphur-containing amino acids are partly oxidized during the ion-exchange procedure or subsequent evaporation of the HCl eluate. Accordingly, Cancalon and Klingman (4) improved the recovery by running the ion exchange under N_2 . However, in the case of phenylalanine, tyrosine, and tryptophane, other reasons for bad recovery should be sought. The unreliable exchange performance of the Amberlite resin can probably be attributed to the influence of the surface area of the resin particles on the reaction rate constant of ion exchange. The ion-exchange capacity is proportional to the surface area, which is small for the Amberlite but large for the Dowex resin. As already shown (41), reduction of the size of the resin particles increases the exchange capacity for amino acids.

Numerous procedures for making amino acids volatile enough for gas chromatography

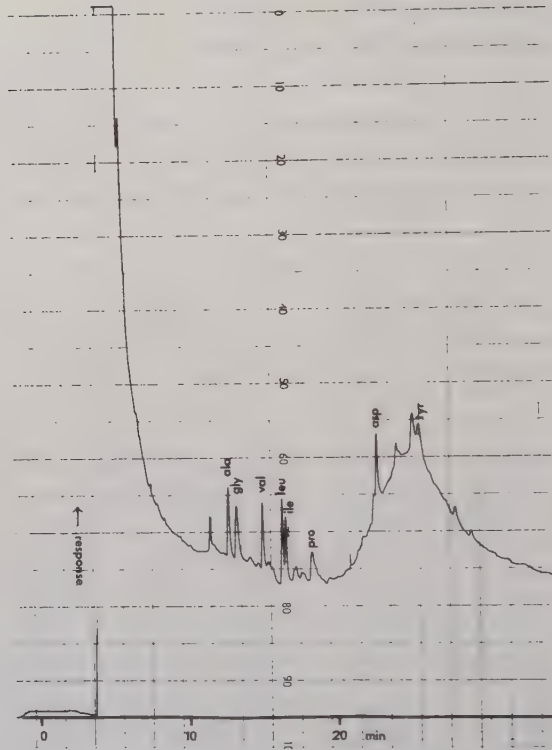


FIG. 8. Glc of leaching amino acids from a beech leaf community. A 25-m glass capillary column supported with OV-101 was used with an initial temperature of 85°C, programming rate, 8°C/min, and final temperature, 215°C. The amino acid peaks represent 20 to 60 ng.

graphic separation have been described. The most common require two steps of synthesis, i.e., esterification of the carboxyl group and acylation of the α -amino and other function groups. Ultrasonic mixing of the medium facilitates direct esterification with acidified anhydrous butanol and propanol. The esters of these alcohols are in particular suitable with respect to volatility (42). March (13) and Moss *et al.* (15,37)

obtained good separation with HFB *n*-propyl esters, but March (13), investigating the reproducibility of response for these derivatives, found that optimal reaction conditions could not be achieved for all amino acids and that a compromise had to be accepted. Aspartic and glutamic acids, when abundant, are difficult to separate from methionine, phenylalanine, and tyrosine using the *n*-propyl derivatives (19). However, the isobutyl

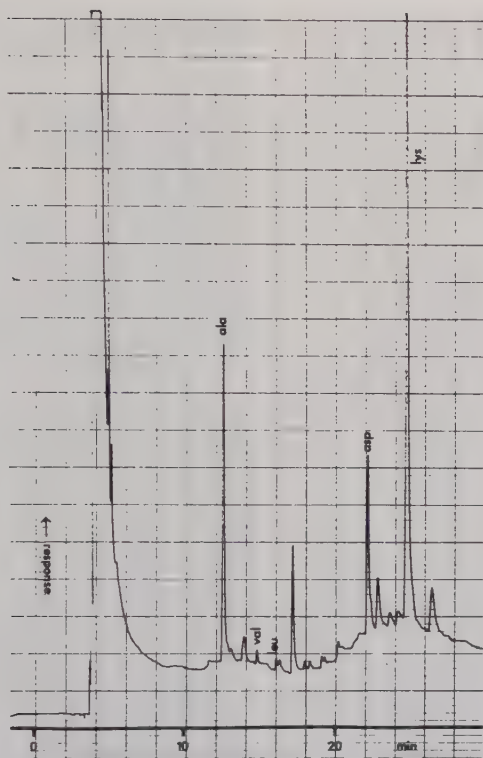


FIG. 9. Glc of amino acids excreted from a laboratory population of the fungi *Arthrobotrys oligospora*. Conditions are as in Fig. 8. The amino acid peaks represent 9 to 4000 ng.

esters present no problem in this respect (e.g., 10). Our finding of better yields with the isobutanol derivatives compared to the *n*-butanol derivatives with a short reaction time, depends most likely on the faster nucleophilic displacement reaction, assumed to be a mixed SN_1 and SN_2 , at the carboxyl group of the amino acid.

The variations in the yields of cysteine and cystine, owing to reduction of cystine to cysteine and oxidation of cysteine to

cystine, have long been a disadvantage of the method. As shown (43), the conversions do not occur on the column but rather during the esterification. The situation can be improved considerably by addition of a reducing agent in the derivatization step (1).

With respect to the concentration of the acid catalyst, Jönsson (7) found that the reproducibility of yields is uncertain at high concentrations and that the solubility of basic amino acids and cystine is low. At



FIG. 10. Identification of valine in a water sample from Stampenbäcken by mass fragmentography. The gas chromatograph was equipped with a glass capillary column coated with OV-101 and the valine peak measured at m/z 268. The peak represents about 50 pg of valine. The first peak after injection is a solvent impurity.

lower concentrations of HCl, the reaction time has to be prolonged. When the concentration of HCl is held between 3 and 4 M, the most satisfactory yield is achieved. The reaction of alcohol and acetylchloride provides a convenient and accurate means of preparing the esterifying reagent and constitutes an excellent alternative to acidifying

the alcohol with gaseous hydrochloric acid. The optimal conditions for esterification found in this work are in agreement with MacKenzie (11).

Jönsson (7) found no significant effect on yield when the acylation time was increased from 10 to 20 min, and March (13) concluded that a deviation by $\pm 10\%$ from 12 min did not affect the yield. In general, a high acylation temperature, e.g., 150°C , gives a more reproducible yield (7,17). Excluding the antioxidant BHT from the acylation step gives unreliable results for methionine and histidine (13), possibly because HFBA has oxidative effects on the derivatives. To remove the final residues of HFBA (to prevent rapid deterioration of the column), a vacuum pump had to be used under standardized conditions, as arginine seems to be partly lost with prolonged evaporation. An important consideration when dealing with natural samples is the behavior of amines and peptides in the process of the determination of free amino acids. Tests were made with phenylalanylvaline, alanylleucin, and glycylaspartic acid. No gas chromatographic peaks originating from the amino acid constituents or from the specific dipeptides were seen. If the peptides were at all derivatized, they possess very long gas chromatographic retention times.

A slight increase in the polarity of the stationary phase by addition of 2% OV-17 (introducing phenyl groups) to OV-101 significantly increased the separation efficiency of the column. Initial difficulties in separating aspartic acid and phenylalanine were overcome, in particular when using the isobutyl ester. In agreement with the general theory, the liquid phase should be fairly polar, but a compromise must be made between analysis time and separation efficiency. Probably a higher proportion of OV-17 can be used without affecting analysis time. The chromatogram is usually run in less than 1 h using a packed column—the peaks have been resolved in 55 min (13), 45 min (38), and less than 30 min (10).

Glass capillary columns allow separation with superior resolution and shorter retention times. For example, a complete separation in less than 30 min has been obtained (2,7). Increasing the temperature-programming rate from 4°C/min to 8°C/min gives only a minor decrease in separation (10).

The improvements in separation by using glass capillary columns are gained at the expense of sensitivity if an injection split is used. When the split ratio is as low as 10:1 (practical limit), the lost sample amounts to 90%. Either a split system with selective venting of the solvent until the first peak appears or a splitless SCOT column appears to be the ideal system for analysis of amino acids.

The minimum detectable limit of the flame ionization detector is exhausted at about 1 ng of individual amino acid HFBA derivatives. A detection limit of 5 ng of amino acids has been reported earlier (18).

As the HFBA derivatives possess high electron affinity, they are very suitable for EC detection. The sensitivity for HFBAmines has been increased more than 100-fold using ECD (20), and Zumwalt (18) used a ^{63}Ni source to detect 1 pg of methionine and 2 pg of cysteine. Our experience with combination of ECD and amino acid analysis is limited to some simple tests of detector response. However, sensitivity to the derivatives was increased 100-fold without optimization of the technique. The main drawback of the ECD detector in amino acid analysis is the difficulty of avoiding a heavy baseline drift caused by the temperature programming. The effect is usually attributed to irregularities in carrier gas flow. Improving the gas chromatographic techniques involving temperature programming of glass capillary columns connected to the ECD detector is an interesting topic for future research in amino acid analysis.

Another approach is presented by Adams *et al.* (2), who applied the NPD detector to amino acid derivatives, thereby enhanc-

ing the sensitivity approximately 80 times compared with the FID detector.

As demonstrated, mass fragmentography constitutes a sophisticated detection system for individual amino acids at levels less than a picogram. It is especially useful in the search for specific amino acids present in small amounts in unknown complex biological samples. The reliability of the analysis at these levels represents points of interest as the amounts determined approach the normal background levels. The problem of contamination during handling has been investigated in detail by Rash *et al.* (44).

The concentrations of amino acids in the lakes are well in accordance with those found in marine surface waters, i.e., of the order of 0.05 to 20 $\mu\text{g/liter}$ (23,45–48). The between-lake variation may be accidental, but it can also be interpreted as the result of a more rapid uptake of amino acids in the more productive lake.

It is not surprising that the concentration is two to ten times less in the watercourse as the sample was taken during the summer when the input of organic matter is limited, while the lake samples are from the autumn when large amounts of excreted organic matter are expected. Provided that histidine and tryptophane found in the running water are produced by leakage from leaves and that their absence in the leaf excretion after 2 weeks is not accidental, a sequence of excreted amino acids from dead-leaf communities can be suspected. This starts with the simple amino acids and terminates with the more complex. The excretion of amino acids from leaf communities can probably supply microbial production in the watercourse. Based on a density of 1 kg of leaves/ m^2 at the river bottom, the average production of each amino acid from 1 m^2 of leaf surface is 2 mg/h [for maximal microbial uptake rates in sea water see (24)].

As has been demonstrated, this method can be applied to an analysis of free amino acids in biological samples of complex ori-

gin with a minimum of sample volume. The advantages obtained can also be used in model laboratory experiments to gain insight into the transport, distribution, and metabolism of free amino acids in biological systems.

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Glass Capillary Gas-Chromatographic Analysis of Free Amino Acids in Biological Microenvironments Using Electron Capture or Selected Ion-Monitoring Detection

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Studies on analysis of free amino acids using a support-coated, open-tube capillary column, and electron-capture detection or selective ion monitoring have been performed on samples from biological microenvironments. For most amino acids the detection limit was found to be less than 1 pg. The preparation of the support-coated open-tube capillary column is described as well as the gas-chromatographic conditions for direct injection and temperature-programmed separation of the *N*-heptafluorobutyl isobutyl ester derivatives. Electron-capture detection and selected ion monitoring are compared with respect to linearity and sensitivity and the bases for the greater sensitivity of electron-capture detection compared with flame-ionization detection using halogenated derivatives is discussed. Applications of the gas-chromatographic method for analysis of free amino acids in environments deliberately chosen very small are demonstrated.

Free amino acids constitute a readily accessible source of nitrogen and carbon for microorganisms, phytoplankton, invertebrates, and higher plants in a wide variety of environments. The nutrient nature of the amino acids is of great importance in the multitude of interactions constantly at play between organisms. Examples of such interactions could be the interplay between soil microorganisms and roots, hyphomycetes and invertebrates, and bacteria and mammals, including man, all of which constitute areas of research of current interest in our laboratory.

Most likely, local concentrations of amino acids in certain environments are more relevant in interactions between organisms than are general concentrations. The rhizosphere—the interface between plant or animal cells and microorganisms—constitutes an example of a microenvironment where amino acids, probably in a stimulatory way, might be expected to affect growth and development. Obviously

the amount of the amino acid material present in such microsystems is extremely small, and the analysis requires detection systems of the greatest possible sensitivity and selectivity.

We recently reported on a micromethod for the analysis of free amino acids in biological systems by gas chromatography using the *N*-heptafluorobutyl (HFB)¹ isobutyl (IB) ester derivatives (1). Various conditions for sample treatment, including derivatization, separation on packed glass columns using OV-101 or OV-17 stationary phases, and flame-ionization detection (FID) were studied in some detail. The detection limit was found to lie in the 1 to 5-ng range

¹ Abbreviations used: HFB, *N*-heptafluorobutyl; IB, isobutyl; FID, flame-ionization detection; SIM, selected ion monitoring; SCOT, support-coated open-tube capillary column; ECD, electron-capture detection; HFBA, heptafluorobutyric anhydride; BHT, 2,5-di-*tert*-butylhydroxytoluene; PBS, phosphate-buffered saline; RMR, relative molar response; EI, electron impact; glc, gas-liquid chromatography.

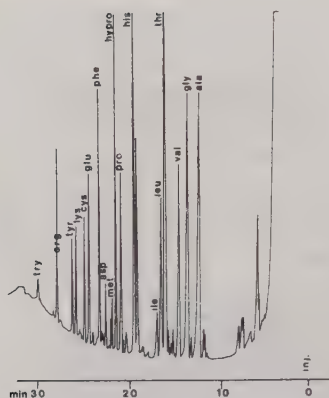


FIG. 1. The glc chromatogram of a reference mixture of 19 protein amino acid derivatives using the SCOT column and FID. Chromatographic conditions are described in the text. Each peak represents 40–125 ng of amino acids.

for individual amino acids. Moreover, it proved possible to carry out quantitative analyses of natural amino acid samples in parts per billion concentrations. Electron-capture detection of the HFB-IB amino acid derivatives increased the sensitivity by a factor of at least 100. Furthermore, we demonstrated briefly the use of the mass spectrometer operated in the selected ion monitoring (SIM) mode as an even more sensitive—and, furthermore, selective—gas-chromatographic detector.

The present communication deals with the preparation and use of a suitable support-coated, open-tube capillary column (SCOT) for direct injection, connected either to the ECD or to the mass spectrometer for detection of amino acids at the femtomole level.

MATERIALS AND METHODS

Reagents. Analytical grade amino acids were obtained from Fluka AG, Switzer-

TABLE I
EFFECT OF ECD ON AMINO ACID RMRs

Amino acid	RMR _{ECD} /RMR _{FID}
Serine	2.0
Histidine	2.5
Hydroxyproline	1.8
Methionine	1.4
Glutamic acid	3.9
Tyrosine	1.4
Tryptophane	6.0

Note. RMRs are calculated for each amino acid relative to norleucine by peak-height measurement. Sensitivity of ECD and FID is compared by using the quotient between RMRs. Quotients >1 are given. RMRs for other amino acids tallied with those given in Ref. (10).

land, and Sigma Chemical Company, St. Louis, Missouri. Stock solutions were prepared in 0.1 M HCl and stored at 4°C. Dowex 50 W (H⁺), 100–200 mesh, heptafluorobutyric anhydride (HFBA) and 2,5-di-*tert*-butylhydroxytoluene (BHT) were from Fluka. HCl, NH₄OH, iso-butanol,

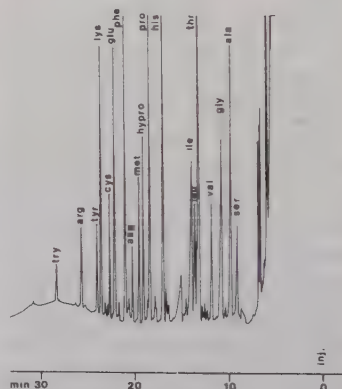


FIG. 2. The glc chromatogram of amino acid derivatives on the SCOT column in connection with EC detection. Chromatographic conditions are described in the text. Each peak represents 200–600 pg of amino acids.

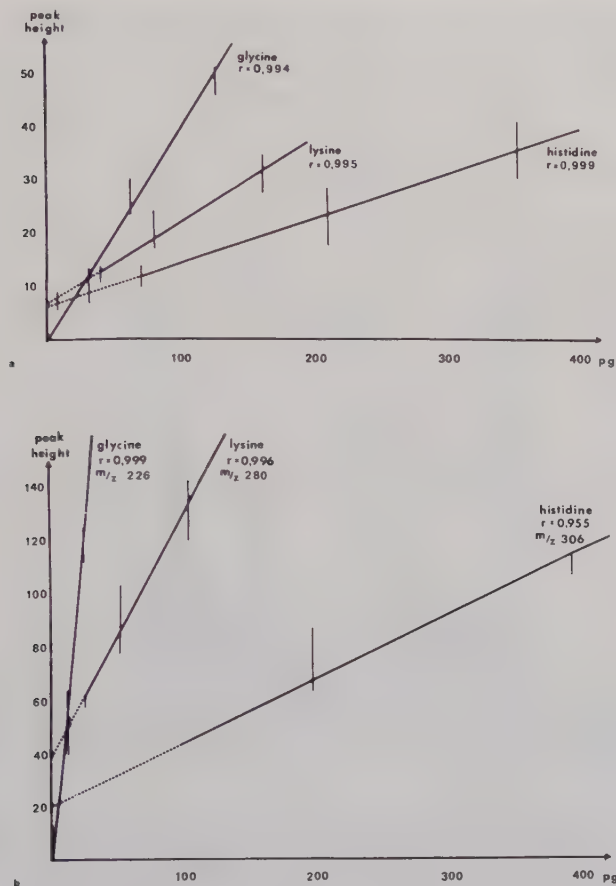


FIG. 3. Peak heights (mean and SD) vs sample size using ECD (a), att. $\times 8$, and SIM (b). Conditions are described in text.

chloroform, dichloromethane, acetylchloride, ethylacetone, acetic anhydride, and carbon tetrachloride were of analytical grade and used without further purification. Silanox 101, 7 μm , was obtained from

Chrompack, Holland. Carbowax 20 M, SE-30; OV-17 and OV-1 were from Alltech Inc.

Sample pretreatment and derivatization. The sample was passed through the ion-exchange column (1), transferred to a Pyrex

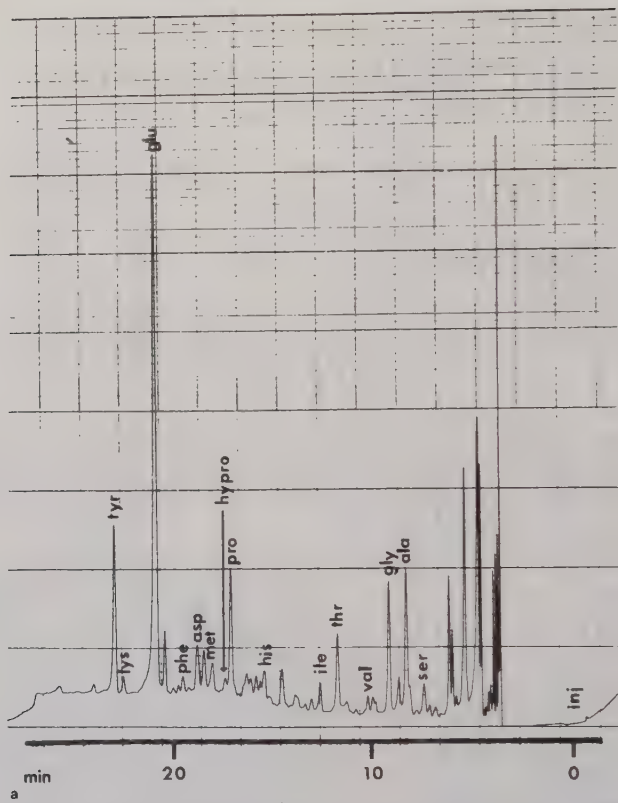


FIG. 4. The glc chromatogram of derivatized amino acids in surface film (a) and subsurface water (b) from lake Skäravatten. The amino acid derivatives were separated on the SCOT column using ECD detection as described in the text (att. $\times 256$). The peaks in (a) represent 50 (hydroxyproline) to 2000 pg (e.g., glycine) of amino acids corresponding to 0.3 to 9.5 ng/cm² surface film. The peaks in (b) represent 30 (lysine) to 400 pg (glycine) corresponding to 0.08 to 1.2 ng/ml subsurface water.

glass capillary tube (80 $\times$ 2 mm i.d.), and evaporated to dryness. Derivatization was performed by esterification with *iso*-butanol 3 M HCl and acylation in HFBA and BHT dissolved in ethyl acetate. After evaporation of excess reagent, the sample was dissolved in 5 μ l ethyl acetate and stored at -20°C

in sealed tubes. Further details of the procedures are given elsewhere (1).

Capillary column preparation. Twenty-five meter soda-lime glass capillaries (i.d., 0.5 mm) were washed with dichloromethane and dried with N_2 at 400°C for 2 h (2). The capillaries were filled with dry hydrogen

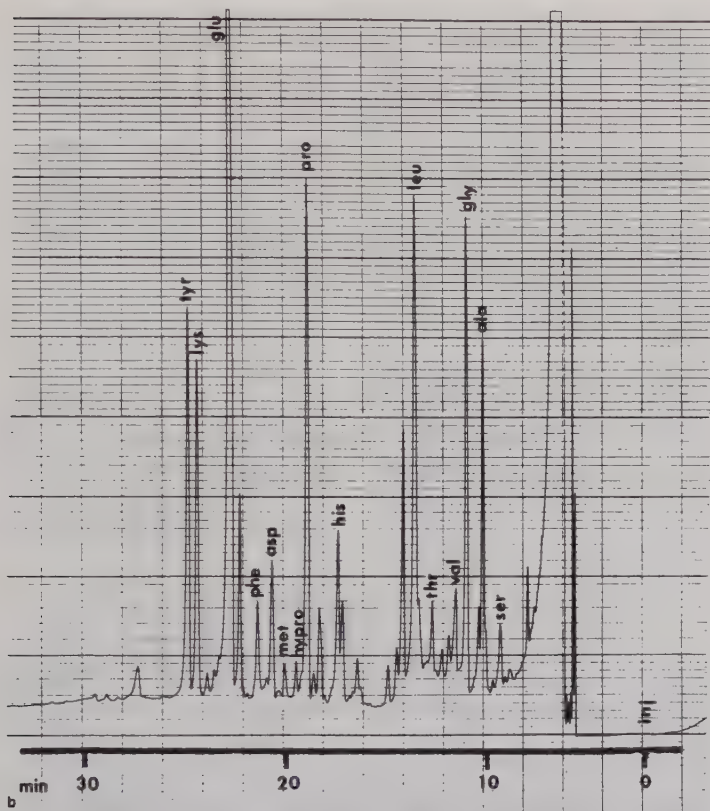


FIG. 4.—CONTINUED.

chloride gas, sealed, and heated at 350°C overnight. The columns were deactivated twice with a solution of 5% Carbowax 20 M in dichloromethane (3), washed with 10 ml dichloromethane, and dried with a stream of nitrogen.

A coating suspension was prepared by dissolving 0.5 g of the stationary phase (SE-30 or OV-17) in 100 ml carbon tetrachloride followed by 2.0 g of Silanox. The

suspension was homogenized in an ultrasonic bath for 15 min. An amount equivalent to about 20% of the column length, preceded by a short plug of chloroform, was forced through the column by nitrogen pressure at a rate of 5–8 cm/s. The solvent was removed with nitrogen, taking 3 h.

The column was then dynamically re-coated using a 2% solution of the stationary

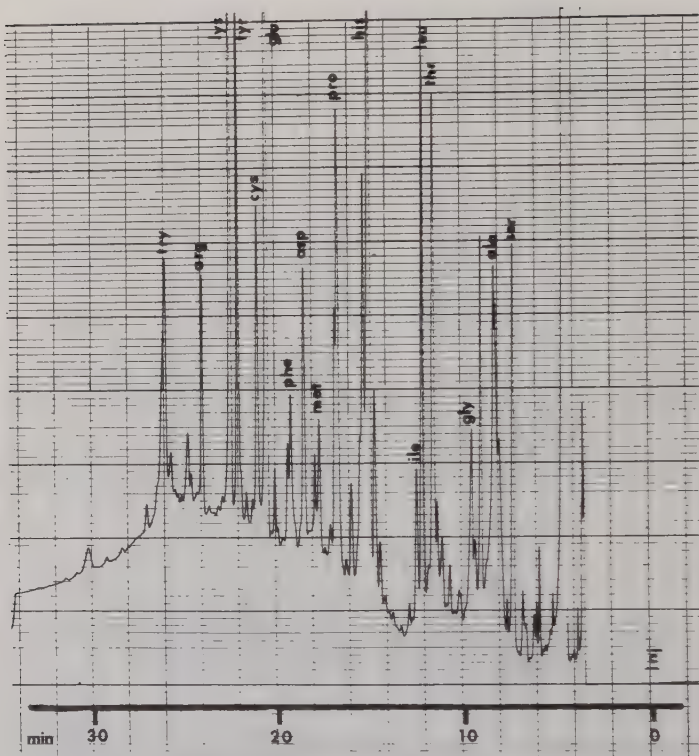


FIG. 5. The glc chromatogram of derivatized amino acids in honeydew from aphids on a black currant leaf. The amounts detected ranged from 130 pg (glutamic acid) to 3 ng (proline) corresponding to a production of 0.7 ng h^{-1} and 16 ng h^{-1} , respectively.

phase in carbon tetrachloride. The plug (25% of the column length) velocity was 2 cm/s and the column was subsequently dried.

Column conditioning was performed with nitrogen as carrier gas (10 ml/min). The column oven was heated at a rate of 0.5°C min from ambient temperature to 200°C and held there for 6 h. The temperature

was then increased at the same rate to 250°C (nitrogen flow 2 ml/min) and held there for 2 h.

Gas chromatography. A Varian Model 3700 instrument equipped with a flame-ionization detector and a ^{63}Ni electron-capture detector was used. Two 25-m capillary columns, coated with SE-30 and OV-17, were coupled together by a piece of shrink-

able Teflon. To prevent deterioration of the capillary column a short, all-glass splitless precolumn (i.d., 0.5 mm) packed with 3 cm of 2% OV-1 on Chromosorb G, 45–60 mesh, was used. The combined SCOT columns were connected to the detector base by a glass-lined tube allowing a make-up gas flow of 20 ml/min. The chromatographic conditions were: injector temperature, 250°C; detector temperature, 290°C. The oven was initially held isothermal at 105°C for 5 min. The temperature was thereafter programmed to rise to a rate of 6°C/min to 220°C. The nitrogen carrier gas flow was 3.5 ml/min.

Mass spectrometry. A Varian MAT-112 GC/MS instrument was used for the mass-fragmentation studies. The combined SCOT columns were installed with an all-glass splitless injection system. The ion source was kept at 250°C and electron energy was 75 eV. Glycine was detected when monitoring the fragments at m/z 226, lysine at m/z 280, and histidine at m/z 306 (4).

SAMPLING

To demonstrate the application of the method, free amino acids were sampled from some microenvironments.

Collection of surface microlayer and subsurface water. Hydrophobic Teflon disks (50.2 cm²) (5) were allowed to touch the water surface. The disks were shaken to remove the adhering water. Extraction of adsorbed amino acids was done with ethanol:water (80:20, v/v) and the mixture was treated as described. Subsurface water (100 ml) was collected at a depth of 0.5 m close to the place where the surface microlayer was sampled.

Honeydew from aphids. Honeydew from an aphid colony on a black currant leaf was allowed to accumulate on a plastic sheet for 12 h. The honeydew was washed from this surface with deionized, distilled water.

Dew of the Lady's mantle. Dew droplets formed on the mantle of *Alchemilla*

vulgaris were collected in capillary glass tubes (i.d., 2 mm). Sampling was made at nightfall as well as at dawn. The dew was washed out of the tubes with deionized, distilled water.

Nematode exudation. Axenically cultivated *Panagrellus redivivus* (6) were washed free from medium by rinsing eight times with distilled water. Individual specimens were transferred to glass capillaries (i.d., 2 mm) with 100 μ l of deionized, distilled water and incubated for an appropriate time. After ensuring that the nematode was still alive, the tube was sealed and stored at -20°C. Care was taken to include only the aqueous phase in the analysis.

Ornithine formation by mycoplasma. *Mycoplasma hominis* was kept in a modified Hayflick medium containing 880 mg l⁻¹ of arginine. An inoculation containing 10² colony-forming units was washed twice with phosphate-buffered saline (PBS) and incubated at 37°C in PBS treated with penicillium (500 IE/ml). Samples (5 ml) were centrifuged after 1–5 h and the supernatant was stored at -20°C.

RESULTS AND DISCUSSION

Separation and Sensitivity

A complete separation of 19 protein amino acids was achieved on the SCOT column used (Figs. 1,2). A difference in the order of elution compared with our previous findings (1) was observed with the combined column. Alanine and glycine were now preceded by serine, which previously was eluted between threonine and leucine. Additional small peaks appeared when picomole injections were made. Some of these peaks represented impurities in the amino acid standards and some of them, probably denote incomplete diacylation of the amino acid. Another source of extraneous peaks is the presence of carbonyl compounds, e.g., butyraldehyde, in iso-butanol, which may condense with the NH₂ group during butylation (7).

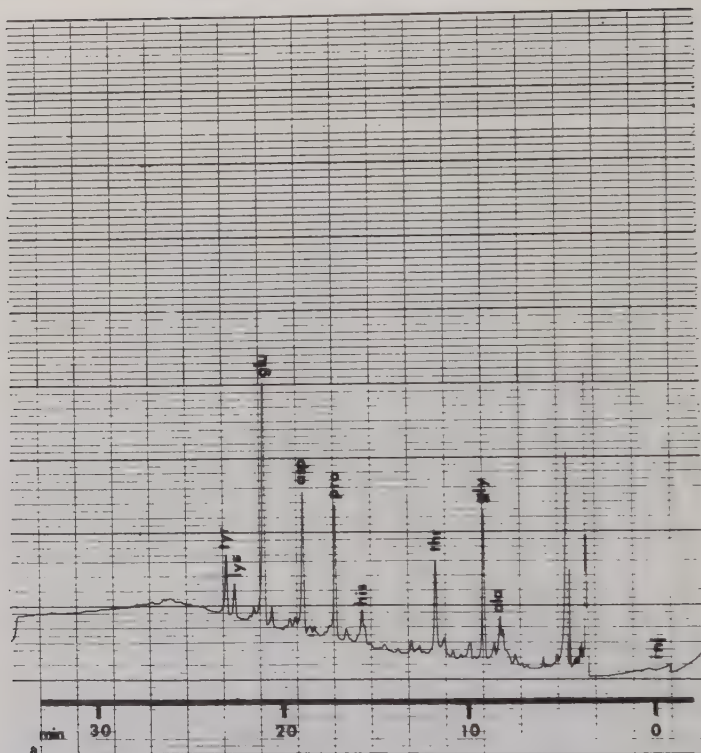


FIG. 6. The glc chromatogram of derivatized amino acids in a dew droplet of lady's mantle from an evening (a) and a morning (b) sample. Whereas the evening droplet contained between 0.5 ng (alanine) and 10 ng (methionine) of amino acids, the amounts had increased to 50 and 500 ng, respectively, in the morning droplet.

A significantly increased sensitivity (generally corresponding to more than three orders of magnitude) toward the EC detector as compared with the FI detector was observed for all amino acids. This was attributed to the high-electron withdrawing capacity of the acyl group which is assumed to depend on the increased polarity of the carbonyl carbon atom induced by the con-

siderable electronegativity of the seven fluorine atoms (8). Diacylation of the molecule as, for example, in the case of serine, tyrosine, histidine, and tryptophane, further increases the sensitivity (Table I). The imidazole and indole ring system of both histidine and tryptophane appeared to give additional ability for electron capturing, resulting in high RMR (molar response

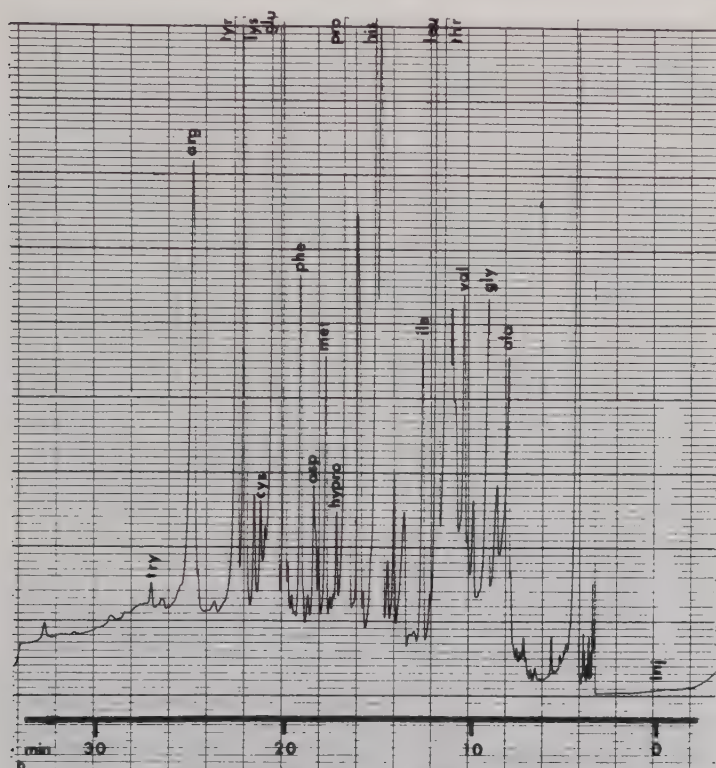


FIG. 6.—CONTINUED.

relative to norleucine) quotients. A difference in sensitivity between the derivatives of the two dibasic amino acids aspartic (quotient < 1) and glutamic acid (quotient 3.9) was noted. The ready formation of the cyclic pyrrolidone carboxylic acid ester from the diester of glutamic acid at the esterification temperatures used (9) could be responsible for this difference. Such ring closures are not known to occur in the case of aspartic acid under the conditions used.

Conversion of arginine to a triacylated derivative, which would yield very high sensitivity, was prevented by the esterification of the guanidino group. This incomplete acylation is reflected in the large standard deviation of its RMR (10).

The linearity of the detector was found satisfactory ($r = 0.99$) within the 10 to 400-pg range (Fig. 3a) and the detection limit was less than 1 pg for most amino acids. This corresponds to a more than 1000-fold

increase in sensitivity compared with the combination of a packed column and FID (1). Significant background amounts of lysine and histidine probably account for the nonzero values.

Most of the amino acid derivatives exhibit intense characteristic ions upon electron impact (EI) at 70 eV (4). In the case of the aliphatic amino acids such ions are formed by loss of the *i*-butoxycarbonyl group from the molecular ion. The hydroxy, cyclic, dicarboxylic, and aromatic amino acids also display characteristic ions usually involving loss of the alkoxycarbonyl and/or acyl groups.

For the mass spectrometric detection studies were chosen glycine, lysine, and histidine. Glycine gave rise to characteristic intense ions (66.1%) at m/z 226 ($M-OCOC_4H_9$), lysine (50.5%) at m/z 280 ($M-(C_5F_7CONH_2 + OCOC_4H_9)$) and histidine (23.8%) at m/z 306 ($M-OCOC_4H_9$). Since the ECD experiments were recorded at reduced amplification (att. 8) it may be concluded that roughly the same sensitivity and reproducibility (coefficient of variation < 0.25) can be achieved when using SIM detection (Fig. 3b) as with ECD detection for the three amino acids tested. The linearity of the plots in Fig. 3 furthermore indicates that quantitative estimations can be made by direct comparison of peak heights.

While the SIM technique is convenient for selective determination of one or a limited number of individual amino acids in the low picogram range, EC detection is better suited to simultaneous analyses of a large number of amino acids in small quantities. The EC detector, however, is significantly more sensitive to column bleed, baseline drift, and sample contamination.

APPLICATIONS

Aquatic Surface Microlayers

Natural waters—whether marine or limnic—have at the air/water interface a very thin layer consisting of enriched organic and inorganic material in which populations of

various microorganisms live. The rich abundance of organic matter in this microenvironment suggests that it is of profound importance for the population dynamics of microorganisms, phytoplankton and zooplankton. Current research has revealed that this layer contains an enrichment of fatty acids, alcohols, triglycerides, chlorinated hydrocarbons, organic and inorganic P and N, and trace metals (11). To our knowledge there is so far no information available about the free amino acid content of the surface microlayers. Both chromatograms in Fig. 4 were dominated by glutamic acid, alanine, glycine, proline, and tyrosine, but a difference was observed in the case of lysine and leucine, which were relatively more abundant in the microlayer than in the subsurface water. An enrichment of total amino acids—corresponding to several orders of magnitude—in the microlayer as revealed by the sampling device was observed.

Honeydew from Aphids

Aphids are known to pierce the host's phloem and extract nutrients which are then released as honeydew, mainly sugars. This subsequently falls to the ground. Although detrimental in a number of ways, aphids are also assumed to be beneficial for the plant by honeydew production (12). Large amounts of amino acids are present in honeydew droplets (Fig. 5). It was calculated that the contribution of amino acids from a single leaf to the soil corresponds to 2 μ g per day. However, this was probably only a fraction of the dissolved nitrogen in the honeydew, as ammonia is usually the predominant nitrogenous waste from aphids (13). The importance of the amino acid release for microbial turnover rate and plant production remains to be assessed.

Dew of the Lady's Mantle

The droplet of lady's mantle is another microenvironment containing both organic and inorganic nutrients. The amino acid con-

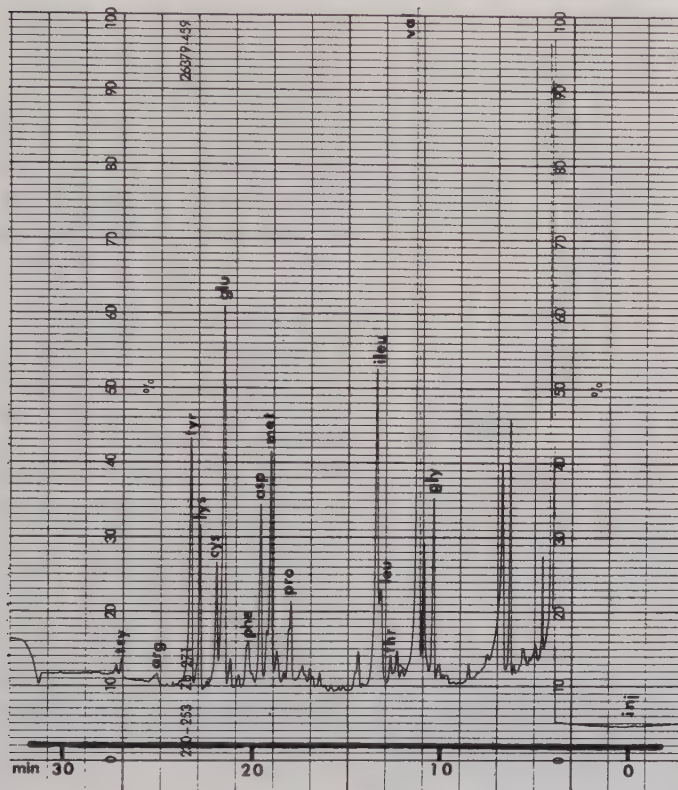


FIG. 7. The glc chromatogram of amino acid exudation from one axenically grown specimen of the nematode *Penagrellus redivivus* incubated for 19 h in deionized water. The peaks represent between 50 pg (threonine) and 2500 pg (valine) corresponding to an exudation rate of 1 and 50 ng h^{-1} . The blank values were nonsignificant.

tent of a freshly formed evening dew droplet (50 μl) (Fig. 6a) was compared with that of a morning dew droplet (50 μl) to which exudation had continued during night (Fig. 6b). A considerable increase in the amino acid content is noted—threonine, leucine, histidine, glutamic acid, lysine, tyrosine, and arginine being the most prominent. The dew droplets thus represent a rich

source of nitrogen readily accessible for bacteria, fungi, and protozoa, which may develop on the plant surface.

Exudation of Free Amino Acids from Nematodes

During studies at this institute on the role of nematodes for the induction of trap formation in the nematode-trapping fungus,

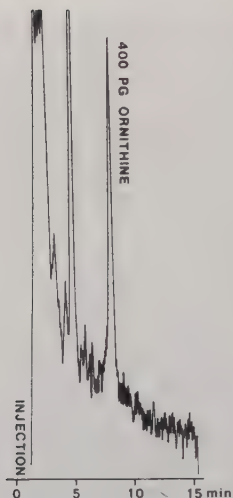


FIG. 8. Mass fragmentography of ornithine produced by *Mycoplasma hominis* during incubation in a phosphate buffer (see text). The peak was measured at m/z 266 and represents 400 pg of ornithine. One-tenth microliter out of 5 μ l of derivative solution was injected.

Arthrobotrys oligospora (6,14), it was considered of interest to investigate whether exudation could be measured on the individual level. Previous investigations had shown that nitrogen containing exudates played an important role in this respect. One individual of axenically grown *Panagrellus redivivus* was therefore allowed to exude into deionized water in a capillary glass tube for varying periods of time. Even after 2 h of incubation, appreciable amounts of amino acids could be detected. After 19 h only one-fivehundredth of the total exudate was needed for the complete analysis (Fig. 7).

Detection of *Mycoplasma* by Monitoring Ornithine Formation

Some species of mycoplasma are known to catalyze enzymatic hydrolysis of arginine

to citrulline and ornithine by ornithine desmidase, a phenomenon which has not been demonstrated in mammalian tissues. Several techniques have been employed to confirm contamination of tissue cell cultures by mycoplasmas. As the detection of mycoplasmas in such cultures using conventional isolation techniques is so tedious and unreliable, attempts have been made to use nonculture methods. In an application of the GC-SIM technique described, the ornithine exuded by *Mycoplasma hominis* after 2 h incubation was selectively examined by using SIM detection monitoring at m/z 266 ($M-(C_3F_7CONH_2 + OCOC_4H_9)$) (Fig. 8).

We believe that the use of capillary-ECD and capillary-SIM gas chromatography opens up possibilities to study the chemical nature of specific interactions in microenvironments. Although this communication concentrates on amino acids, the possibility of using ECD to detect halogenated derivatives of other important types of exudation products, e.g., saccharides and alcohols, should be pointed out. Furthermore, in the case of SIM detection, high degrees of sensitivity and selectivity can be achieved also with underivatized compounds, such as volatiles, and for derivatives without halogens. Further detailed studies on the chemical composition of microenvironments in the rhizosphere and on the chemical basis of interactions between cell surfaces are in progress at this institute.

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COMPENSATION OF BASELINE DRIFT IN TEMPERATURE PROGRAMMED

CAPILLARY GAS CHROMATOGRAPHY WITH

ELECTRON CAPTURE DETECTION

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Reduction in standing current of an electron capture detector due to bleeding from the stationary phase is one of the major problems in temperature programmed GC-ECD (Pellizzari 1974). The reduction may emerge from trace contaminant vapors in the carrier gas or pyrolysis products of the stationary phase. Bleeding increases exponentially with temperature (Devaux and Guiochon 1967), producing a dramatically rising baseline at higher oven temperatures, which can make quantification of peaks difficult. Use of nonpolar phases reduces bleeding effects, while high molecular weight compounds with large electron affinities may cause severe baseline drift with polar phases.

The conventional approach to compensate for baseline drift, dual column analysis, is usually not practised in capillary GC-ECD due to costs of duplicating detector and column. Recently, a single column compensation method, that stores the column bleed profile during a blank run, was presented (Altmayer and Snyder 1980). We here report on a simple displacement of the electrometer voltage to compensate for baseline drift in capillary GC-ECD.

The experiments were performed with a Varian GC 3700 equipped with a ^{63}Ni detector. The baseline compensation was achieved by utilizing a voltage in the automatic linear temperature programmer (ALTP), Fig.1. The voltage, which is proportionally decreasing with increasing temperature, was inverted and amplified so that it cancelled out the drift caused by temperature programming when fed into the electrometer. The slope control sets the gain for a correct amount of compensation and the zero control is for zeroing at the initial temperature. With the On/Off switch the circuit can be switched out when no compensation is required.

The performance of the compensation mechanism was demonstrated with a sample of amino acids, which represent a class of compounds with a wide range of boiling points and consequently requiring temperature programmed separation. Due to the low volatility of amino acids suitable derivatives have to be prepared for gas chromatography. After cleanup by ion-exchange, the amino acids were converted to their corresponding heptafluoroisobutyl derivatives (Bengtsson and Odham 1979). Those are highly electron absorbing and volatile and offer a potential to analyze amino acids at the trace levels found in biological microenvironments (Bengtsson et al. 1981).

Derivatives were separated on a 50 m SE30/OV17 SCOT column, i.d. 0.5 mm. Oven was held isothermal at 105°C for 5 min, then

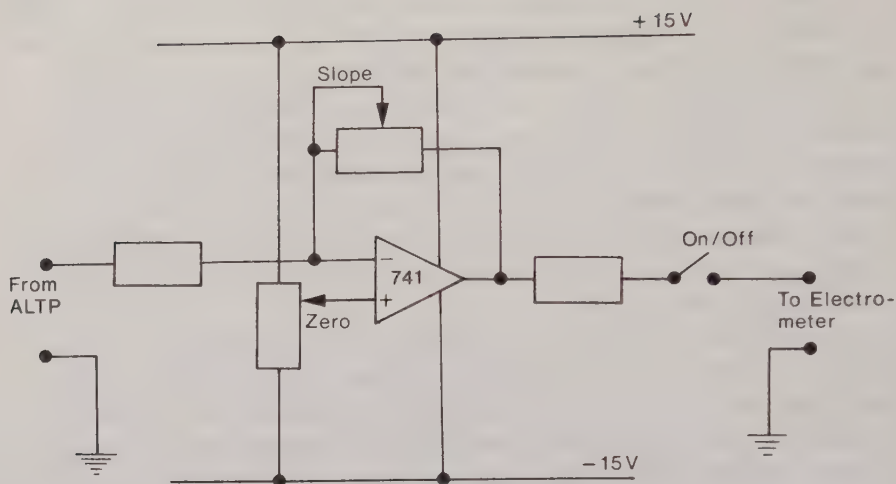


Fig.1. Schematics for a linear compensation of baseline drift.

programmed at $6^{\circ}/\text{min}$ to 220° and held there for 10 min. Injections were made splitless at 250°C . N_2 was used as a carrier gas at 4.5 ml/min and the ECD was held at 300°C . 0.5 μl was injected, representing approximately 0.76 pmole for the large peaks.

At the attenuation and range used, 16×10^{-10} amp/mV, the uncompensated analysis shows a heavy bleed and the baseline runs off the scale (Fig.2a), while the compensation keeps the baseline in its starting position (Fig.2b), thus facilitating integration of peaks.

The compensation method was successfully applied to samples with less than 10 fmole substance but at even less levels a baseline hump between 110°C and 150°C could not be avoided if the last eluted peaks were not to be overcompensated and appearing below the baseline. This effect was certainly due to the exponential relationship between bleeding and temperature. For these cases an exponentially compensating voltage may be applied to the electrometer.

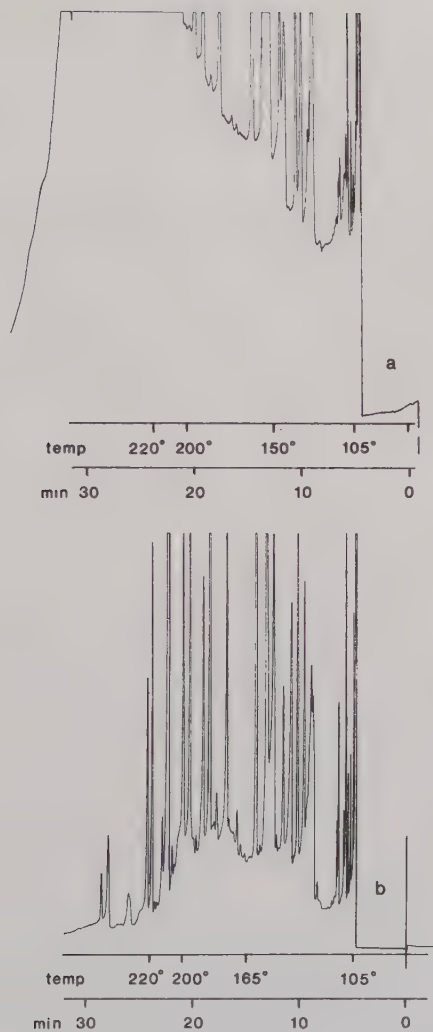


Fig.2. Gas chromatogram after injection of a 0.5 μ l sample of amino acids on a 50 m SCOT column, temperature programming with a ^{63}Ni detector. Without baseline correction (a) and with baseline correction (b).

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SWITCHING FOR AMINO ACIDS IN AQUATIC HYPHOMYCETES

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Many stream communities are primarily supplied with energy and nutrients from decomposing leaves. The decomposition rate depends on the chemical composition of leaves, on environmental parameters such as temperature and pH, and on the colonization rate of microorganisms and small invertebrates. The utilization of amino acids in leaf protein and leaf leachate by aquatic hyphomycetes was studied during decomposition in a combined field and laboratory experiment. Leaves were sampled from a stream which exhibited a seasonal variation in free amino acid concentration in surface water, reaching peaks in fall and winter. In the leaf drift environment the concentration of amino acids was approximately two orders of magnitude higher than in surface water. Protein amino acid content, which was higher in alder leaves than in beech leaves, decreased exponentially and faster in alder leaves, so that protein amino acid content was similar in the two leaf types after 9-10 weeks decomposition. From 55 to 75 % of leaf amino acids were used instantaneously by attached fungi, which grew well, especially on alder leaves, regardless of the presence of a grazing amphipod. If nitrogen was a limiting nutrient source for fungi, it appeared to be more advantageous to colonize alder leaves. Four times more fungal species were found on alder leaves than on beech leaves. The change in concentration of amino acids in leaves and water was described by a set of differential equations. Rate constants for the transfer of amino acids from leaves and water were estimated by experimental data and the preference in fungi for protein bound and free amino acids evaluated.

The amounts of free amino acids in water absorbed by fungi varied between leaf types and leaves at different stages of decay. Experimental data showed a switching behaviour in fungal absorption of dissolved amino acids so that absorption became superproportional at a certain proportion of free amino acids available in the water.

INTRODUCTION

The size and pattern of energetic pathways and the mobilization and utilization of low molecular weight, biologically active chemical compounds in woodland streams is ultimately controlled by the rate of decomposition of allochthonous organic material (Cummins et al. 1966, Minshall 1967, Hynes 1970, Fisher and Likens 1972, 1973, Cummins 1974), the main source of which is autumn-shed leaves (Kaushik and Hynes 1968, Petersen and Cummins 1974). By processing the particulate and dissolved organic matter the stream community receives 50-99 % of its total energy requirement (Cummins et al. 1966, Fisher and Likens 1972). More than 80 % of the allochthonous energy is usually transformed by microorganisms (protozoa, bacteria, fungi) (Minshall 1967, Fisher and Likens 1972, Cummins 1974), which are important intermediaries in the stream detrital food chains.

A rapid leaching of water-soluble, low molecular weight substances (e.g. sugars, amino acids, aliphatic acids) has been observed from leaves (Nykqvist 1963, Wetzel and Manny 1972), but few attempts have been made to separate the individual chemical com-

pounds. Changes in concentrations of chemical components from leaves generally conform to a negative exponential decay model (cf. Nykvist 1963, Kaushik and Hynes 1971, Iversen 1973, Suberkropp et al. 1976).

The early stages of leaf decay are dominated by hyphomycetes (Kaushik and Hynes 1968, 1971, Suberkropp and Klug 1976). Many aquatic hyphomycetes produce extracellular enzymes for decomposition of major structural components of the leaves. At least part of the exploited substrate is thus metabolized and absorbed by the fungi, but since extracellular enzymes are involved, some of the products of degradation may escape (Nykvist 1963, Bärlocher and Kendrick 1974).

The mycelium on and in the leaf is preferentially grazed by leaf-eating animals (Bärlocher and Kendrick 1973, 1976, Kostalos and Seymour 1976), which probably select mycelium-rich leaves due to their higher nutritional value. It is not known if the stream animals also assimilate dissolved organic matter released from the leaves by fungal activity.

The purpose of the present work was to study the hydrolysis of leaf proteins and subsequent absorption of free amino acids by aquatic hyphomycetes on two different types of leaves, alder and beech leaves. A model for transport of amino acids was tested in a laboratory experiment in which leaves were colonized by micro-organisms and invertebrates present in a woodland stream.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Decaying leaves of alder Alnus glutinosa L. and beech Fagus sylvatica L. were picked in autumn 1977 from trees close to Stampenbäcken, a small stream in southern Sweden (Hulthin 1971). Six leaves were enclosed in 2 m/m mesh nylon bags (20x10 cm). The bags were attached to wood stakes by nylon strings in the stream close to the trees. After two days the bags were moved to the laboratory. For the experiments, 279 alder leaves and 180 beech leaves were used.

The surface of each leaf was lightly cleaned with a brush under cold tap water for one minute. The leaves were incubated in cold sterile filtered tap water for four hours to ensure that initial rapid leaching did not affect the experiment. Three

leaves were then placed in a glass jar, which was filled with sterile filtered cold tap water (300 ml) and incubated at 4°C (Heto refrigerator). Thirty jars were set up for each leaf species. The same number of jars was supplemented with three surface sterilized leaves (leaves treated for 5 minutes with 0.01 % HgCl_2 followed by washing with filter sterilized cold tap water. Six jars were sampled after 1, 4, 7, 21, and 56 days.

Fifteen jars, each with three alder leaves, were treated with 10 μM lincomycin and erythromycin to remove bacteria. Triplicates of these jars were sampled each time for bacterial determination. Blanks (six jars with sterilized water) were randomly placed in the refrigerator and incubated for 56 days. All jars were connected to an air compressor by acid-washed PVC-tubes and a 0.22 μm Millipore disposable filter, and gently aerated.

Specimens of Gammarus pulex L. (Amphipoda) were sampled in the same stream by electro fishing. Prior to the experiment the specimens were kept at the laboratory in an aerated aquarium supplied with leaves. Eighteen jars were provided with alder leaves. Five animals were then rinsed in tap water and released into each jar. After 1, 4, 7, 14, 28, and 49 days three jars were sampled.

The samples collected were analyzed for free amino acids in water surrounding leaves, amino acids in leaves, length of hyphal mycelium and species frequency, and viable counts of bacteria.

The leaves were removed from the jar, the sample water volume was determined, the leaf-surface was carefully washed with distilled autoclaved water, and the washings were added to the sample. The leaves were briefly dried on a filter paper and weighed. A subsample of the leaves was dried at 85°C and then weighed, and another subsample was used for determination of fungi and bacteria. Remaining leaf material was stored at -20°C for amino acid analysis.

For the second part of the experiment alder leaves were enclosed in the nylon bags and left in the stream for 2, 4, 10, and 25 days. Twelve leaves were used in each bag and four bags were sampled each time. Approximately 500 leaves of each species were taken from leaf drifts in the stream and immersed in aerated cold, filter sterilized tap water in five 10 liter jars.

For each batch of leaves from the stream six leaves were taken for protein analysis. Another six leaves were each provided with 100 ml filter sterilized leachate from the stock of the leaf

drift leaves. Leachate samples were taken after 1, 5, 10, and 20 days, filter sterilized and stored at 4°C. A subsample was analyzed for free amino acids and eventually the concentration was adjusted with either leachate (to increase the concentration) or filter sterilized stream water, to four levels, based on the first experiment. Thus there were four concentrations of free amino acids in the leachate for each batch of leaves.

Another twelve leaves from each batch were incubated in 100 ml filter sterilized tap water. After two days, two leaves and corresponding water samples were analyzed for amino acids. The water in another two jars was exchanged with new filter sterilized tap water and the leaves and corresponding water samples were taken for analysis after two more days. At the same day, the water in another two jars was exchanged with fresh tap water. This procedure was repeated six times during twelve days. The purpose was to estimate the proportion of hydrolyzed proteins not used by the fungi but released into the water, and whether that proportion was related to the concentration of free amino acids in the water.

Water from the six incubated jars with leaves was collected and analyzed for free amino acids every second day during 10 days. The collected water was replaced with fresh water from the same incubated leachate sample and subsample was taken for free amino acid analysis. After 10 days the leaves were used for protein analysis.

AMINO ACID ANALYSIS

Water samples were filtered through a 0.45 µm filter (mixed cellulose acetate and nitrate) to remove particulate matter, and evaporated in a rotary evaporator. The residue was analyzed for free amino acids by a gas chromatographic technique (Bengtsson and Odham 1979).

Surface water samples were collected eight times a year from three sites in Stampenbäcken, one in the upstream section, coinciding with the site for leaf preincubation, one in the middle section of the stream and one downstream. One liter water samples were collected in a PVC bottle and kept frozen until filtered through a 0.45 µm Millipore filter and analyzed for free amino acids. At the upstream site a 10 ml water sample was withdrawn from the interior of a leaf pack by connected a 10 ml syringe to a teflon tubing.

Protein and otherwise bound amino acids were determined in 0.4 g of wet leaf material and in 50-100 μ g fungal hyphae that were separated from the leaves by a scalpel. The sample was weighed in a test tube and 10 ml 6 M HCl added. The tube was flushed with N₂ and sealed. Hydrolysis was performed at 110°C for 24 hours. The amino acid content was then determined as above.

ESTIMATION OF HYPHAL LENGTH

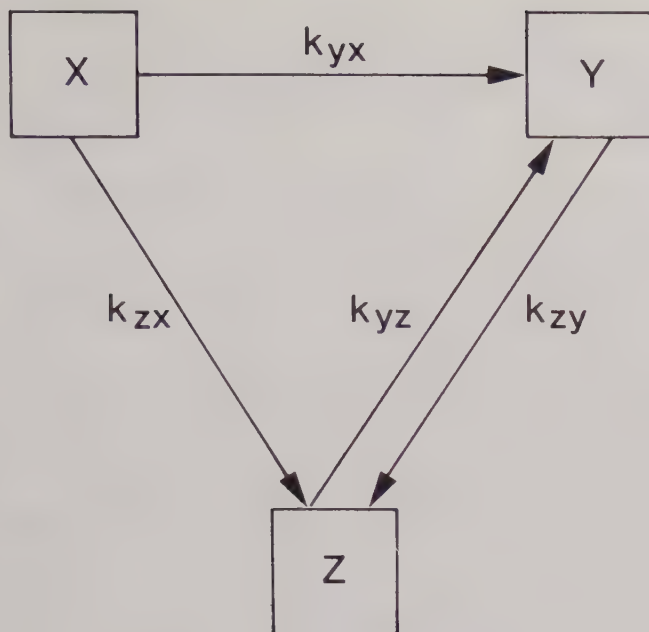
The length of fungal hyphae on leaves was estimated using a slightly modified version of the agar film technique described by Bååth and Söderström (1977). Subsamples, covering a section of at least one vein, were cut out from each leaf in a sample bottle. Subsamples were combined to give a sample wet weight of 1 g. The leaf tissue was macerated in water for five minutes, five ml of the suspension was diluted with a molten 2 % agar solution, a drop was cooled in a 0.1 mm deep, preheated haemocytometer and the solidified agar film was mounted and stained with phenolic aniline blue on a glass slide. From each sample three preparations were studied and ten fields of each were examined with a phase-contrast (X 480) microscope. In each field the number of hyphal cross sections was counted.

Fungi were isolated from leaves preincubated in the stream, basically according to Webster (1959) and Bärlocher and Kendrick (1974). Details are reported elsewhere (Bengtsson subm.). Leaf types were compared with respect to their fungal communities by expressing the frequency of each species in all samples of respective leaf types.

For bacterial determinations subsamples were cut from the leaves, combined and homogenized for five minutes in sterilized water. After appropriate dilution of the homogenate, 0.1 ml of each dilution was spread onto plates of leaf extract agar. Plates were incubated at room temperature for five days and bacterial colonies counted.

THEORETICAL ASPECTS

A theoretical model was constructed to describe the transfer of amino acids in a steady state system including leaves, water and fungi (Fig.1a). The model assumes that fungi process amino acids



1a

Fig.1a. Model describing possible routes for the amino acid transfers close to a leaf surface. X, Y and Z represent concentration of amino acids in leaves, water and fungi, respectively, and k_{ij} are rate constants for the transport into compartment i from compartment j (time^{-1}).



1b

Fig.1b. Simplified model of the interaction between leaves, fungi and water. Symbols as in Fig.1a.

from both leaves and water but also exude amino acids into the water. The X and Y compartments were chosen for monitoring the dynamic change of the amino acid concentration in the model and fungal cells (Z) were assumed to have a constant total amino acid concentration.

If the enzymatic processing of leaf proteins contributes a surplus of amino acids to the water environment, and if hyphomycetes absorb amino acids from the water, examination of Y as a function of time should reveal not only the variation of enzymatic surplus of different amino acids but also the extent to which fungi absorb and utilize amino acids from the water in the presence of leaf amino acids. In a more general sense, the model would allow application and testing of the theory of the optimal foraging strategy (MacArthur 1972) on a process regulating the decomposition of leaves in a stream. The interaction between leaves, fungi and amino acids could thus be described by a simplified model (Fig.1b).

RESULTS

THE MODEL

The change in the concentration of amino acids as a function of time was written as a set of differential equations:

$$\frac{dX}{dt} = -k_{yx}(X-a)$$

$$\frac{dY}{dt} = k_{yx}X - k_{oy}(Y-c)$$

where $\frac{dX}{dt}$ and $\frac{dY}{dt}$ are the rates of change of the concentration of amino acids in leaves and water, respectively, and a and c are asymptotic values. Integrations yielded:

$$X = X_0 e^{-k_{yx}t} + a \quad (1)$$

$$Y = p_1 e^{-k_{oy}t} - p_2 e^{-k_{yx}t} + c; \quad p_1 - p_2 + c = 0 \quad (2)$$

where p_1 and p_2 are coefficients of the exponential terms.

Data for the various compartments were fitted to the model and the best values for the parameters were determined by the method

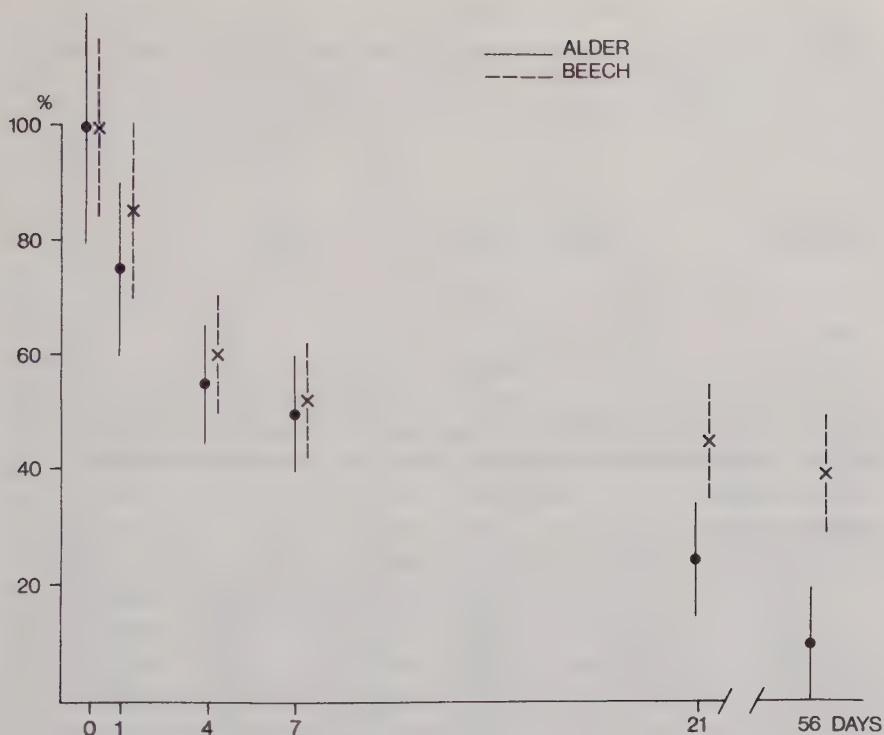


Fig.2. Changes in concentration of total amino acids in alder and beech leaves in percentage of the concentration at $t=0$.

of minimization of the sum of squares of deviations between observed and calculated values.

AMINO ACIDS IN LEAVES AND FUNGI

The release of amino acids from the leaves was adequately described by the model (residual sum of squares 4.87 and 6.14 for alder and beech leaves, respectively).

After a couple of weeks incubation the concentration of protein bound amino acids was reduced to 50 % (Fig.2). The pre-leached alder leaves had more than three times higher concentration of amino acids compared to beech leaves but the difference was levelled out after two months incubation (Tab.1).

TABLE 1

Total concentration (mg/g wet weight) of protein amino acids in alder and beech leaves during incubation in water.

Incubation days	0	1	4	7	21	56
alder	13.68	10.15	7.65	6.95	3.14	1.68
beech	3.81	3.25	2.34	2.02	1.69	1.52

TABLE 2

Concentration of free amino acids ($\mu\text{g/g}$ leaf tissue) in water surrounding alder and beech leaves incubated in water. Mean ($n=6$) and standard deviation are computed for each amino acid. Values are corrected for blanks.

Incubation days		1	4	7	21	56
alder	ala	25+6	106+55	916+50	52+7	4+3
	gly	8+6	49+7	69+39	17+8	5+1
	val	10+7	13+6	97+7	20+5	12+4
	thr	16+6	129+25	357+56	11+1	3+3
	ser	12+6	35+19	57+52	48+33	52+8
	leu	24+0	374+32	270+98	14+7	24+4
	ile	15+8	208+57	189+56	6+4	3+2
	pro	13+6	143+26	108+8	23+6	78+8
	met	6+4	33+23	42+7	57+6	31+4
	asp	20+2	71+27	80+9	76+6	42+6
	phe	51+3	201+4	98+8	27+5	16+8
	glu	20+9	810+87	59+7	5+2	4+3
	tyr	109+16	233+52	250+39	22+3	45+5
	lys	156+67	1077+97	452+96	113+51	29+15
	arg	73+7	61+27	113+46	41+29	26+5
	his	45+6	167+68	143+9	13+9	15+9
	try	34+8	502+56	240+34	22+3	24+3
beech	ala	23+7	36+19	65+23	40+14	37+15
	gly	6+1	10+9	49+31	36+8	18+4
	val	3+2	8+4	87+16	34+6	23+11
	thr	8+6	15+11	209+28	186+17	174+21
	ser	10+4	65+22	76+18	84+13	13+8
	leu	3+2	43+13	83+24	26+11	9+3
	ile	n.d.	n.d.	n.d.	n.d.	n.d.
	pro	18+9	36+7	35+8	12+8	12+6
	met	4+4	14+8	28+16	23+3	54+14
	asp	14+7	44+11	115+22	97+16	53+6
	phe	146+31	187+65	243+35	94+22	87+13
	glu	190+24	397+85	62+17	41+9	34+6
	tyr	20+6	36+16	80+6	46+12	48+17
	lys	36+17	508+44	923+79	168+27	301+46
	arg	68+33	181+62	315+84	18+7	23+8
	his	271+29	37+14	63+16	94+32	124+14
	try	100+69	154+36	156+28	159+18	151+23

The concentration of amino acids in HCl hydrolysate of fungal hyphae obtained from the leaves was virtually unchanged during incubation. The total concentration varied between 3.6 and 4.0 mg/g wet weight with alanine, leucine, glutamic acid and aspartic acid as most abundant.

AMINO ACIDS IN THE WATER

The concentration of free amino acids in the experimental water changed in a way that was predicted by the model (residual sum of squares 11.43 - 15.28). The concentration first increased as the leaves were depleted for amino acids, reached a peak within one week, and then slowly decreased towards an asymptote (Fig.3). During the first week, protein hydrolysis produced and maintained a free amino acid excess, that disappeared as a consequence of reduced protein amino acid supply (cf. Fig.2) and increased absorption. The relative absorption efficiency was higher in the alder fungal community compared to the beech fungal community. The rate constant, k_{oy} , was four times larger and the asymptote value three times lower in the equation describing the change in free amino acid concentration in water with alder leaves compared to beech leaves (Fig.3). The mechanism for the rapid and efficient absorption of free amino acids by the alder leaf community was explored in the second experiment described. The amounts absorbed by alder fungi were comparatively larger, since higher absolute concentrations of free amino acids were found in water surrounding incubated alder leaves compared to beech leaves (Tab. 2). As the incubation proceeded relatively higher concentrations of amino acids were detected in beech leaf water. Lysine was found to be the most abundant amino acid released from both kinds of leaves.

The presence of the amphipod on the alder leaves seemed to slow down the decomposition of leaf protein, and reduced the absorption efficiency of the alder fungi to the same level as the beech fungi (Tab.3, Fig.3.). Notably, the more complex amino acids (tyrosine, lysine, arginine and histidine) were found in relatively higher concentrations in the water as the incubation proceeded.

To ensure that the concentration of free amino acids in the experiment and in a natural environment was similar, a comparison was made with data from Stampenbäcken. Stream water concentra-

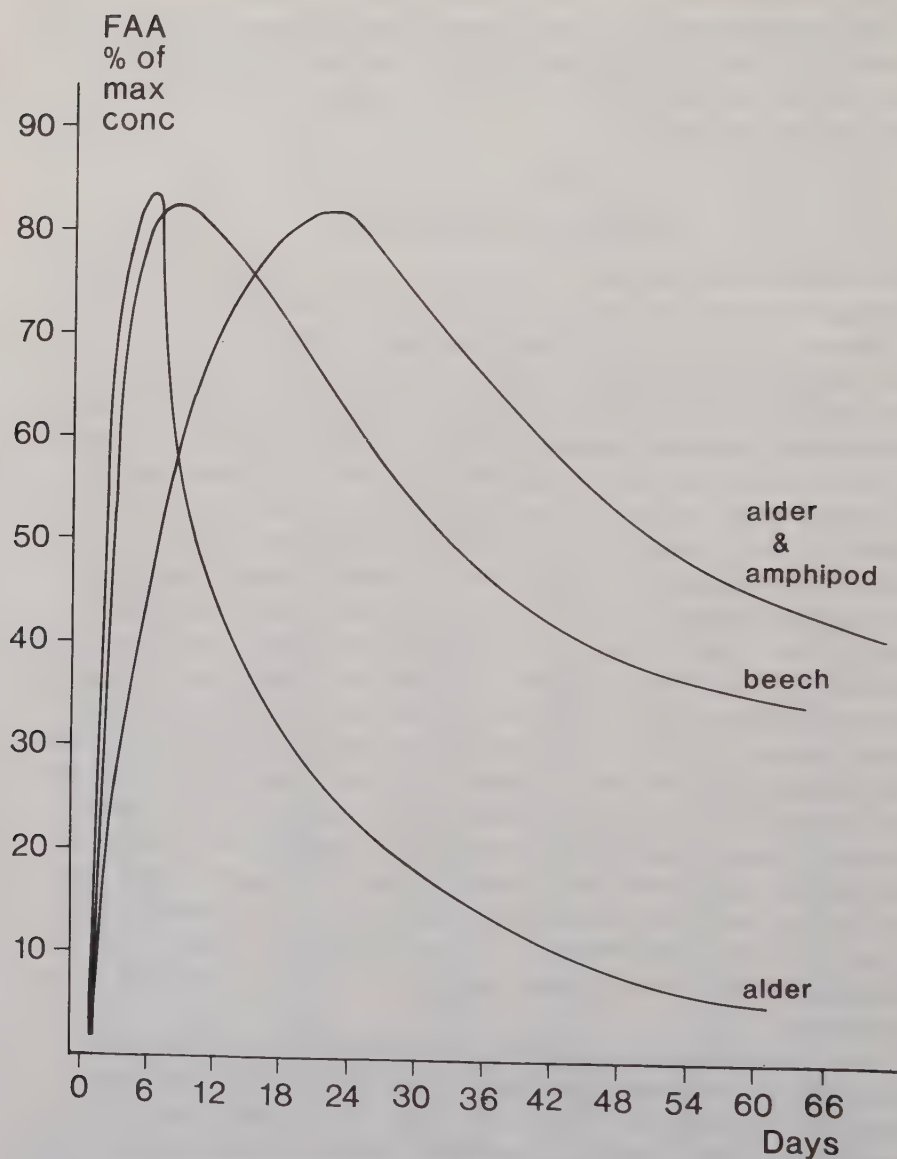


Fig.3. Changes in concentration of free amino acids in water surrounding alder and beech leaves. For each amino acid, means in Tabs. 2 and 3 are expressed in percentage of the maximal mean concentration and these data treated in the model described. The graphs show the computed result of several iterations. The equations corresponding to the different curves are $y=283 \exp-0.12t - 287\exp-0.25t + 3.4$ (alder), $y=93\exp-0.03t - 103 \exp-0.32t + 10$ (beech), $y=277\exp-0.04t - 287\exp-0.08t + 10$ (alder+ amphipod).

TABLE 3

Concentration ($\mu\text{g/g}$ leaf tissue) of amino acids in water surrounding alder leaves incubated in sterilized tap water at 4°C with the amphipod Gammarus pulex L. Mean ($n=3$) and standard deviation are computed for each amino acid. Values are corrected for blanks.

Incubation days	1	4	7	14	28	49
ala	nd	56 $\pm$ 8	67 $\pm$ 13	59 $\pm$ 6	27 $\pm$ 11	6 $\pm$ 4
gly	nd	3 $\pm$ 2	7 $\pm$ 4	6 $\pm$ 3	9 $\pm$ 4	3 $\pm$ 1
val	4 $\pm$ 3	7 $\pm$ 4	9 $\pm$ 5	6 $\pm$ 3	6 $\pm$ 2	3 $\pm$ 2
thr	16 $\pm$ 7	29 $\pm$ 8	78 $\pm$ 17	172 $\pm$ 21	56 $\pm$ 13	7 $\pm$ 3
ser	15 $\pm$ 4	8 $\pm$ 4	11 $\pm$ 6	17 $\pm$ 4	21 $\pm$ 6	25 $\pm$ 10
leu	5 $\pm$ 3	10 $\pm$ 4	9 $\pm$ 5	15 $\pm$ 6	34 $\pm$ 11	46 $\pm$ 22
ile	nd	nd	nd	7 $\pm$ 4	41 $\pm$ 18	44 $\pm$ 24
pro	21 $\pm$ 8	16 $\pm$ 9	18 $\pm$ 7	124 $\pm$ 31	33 $\pm$ 16	66 $\pm$ 19
met	14 $\pm$ 9	78 $\pm$ 21	63 $\pm$ 18	25 $\pm$ 13	29 $\pm$ 17	43 $\pm$ 16
asp	73 $\pm$ 25	48 $\pm$ 13	36 $\pm$ 20	28 $\pm$ 16	31 $\pm$ 12	27 $\pm$ 6
phe	104 $\pm$ 36	697 $\pm$ 109	843 $\pm$ 66	140 $\pm$ 61	80 $\pm$ 26	160 $\pm$ 63
glu	3 $\pm$ 2	5 $\pm$ 3	16 $\pm$ 7	310 $\pm$ 26	158 $\pm$ 67	95 $\pm$ 23
tyr	6 $\pm$ 4	8 $\pm$ 4	27 $\pm$ 13	55 $\pm$ 11	116 $\pm$ 40	124 $\pm$ 35
lys	52 $\pm$ 16	301 $\pm$ 96	1039 $\pm$ 126	1841 $\pm$ 126	572 $\pm$ 88	430 $\pm$ 75
arg	32 $\pm$ 14	40 $\pm$ 12	770 $\pm$ 82	294 $\pm$ 58	46 $\pm$ 18	160 $\pm$ 63
his	31 $\pm$ 11	45 $\pm$ 16	71 $\pm$ 18	66 $\pm$ 21	61 $\pm$ 19	63 $\pm$ 35
try	39 $\pm$ 15	27 $\pm$ 11	8 $\pm$ 6	82 $\pm$ 15	52 $\pm$ 6	49 $\pm$ 16

tions of free amino acids showed a distinct seasonal pattern at all three sampling sites (Tab.4). A peak was found in late autumn and winter, while concentrations declined through spring and summer. The data showed no general trends from the upstream to the downstream sites but concentration of several amino acids decreased with distance throughout the year, indicating heterotrophic utilization. In general, the most abundant amino acids were alanine, glycine, serine, proline, methionine, arginine and histidine. With two exceptions, glycine and serine, the same amino acids were prominent in leachate from either alder or beech leaves or from both of them, and it might be proposed that leaf processing is a main source for these abundant amino acids in the stream. By multiplying the data in Tab.2 by 9^1 , a comparison can be made with data in Tab.4. It is obvious that the concentration of several amino acids was higher in the experiment than in the stream water. However, the concentration of free amino acids in the leaf drift was approximately two orders of magnitude higher than in the surface water and considerably higher than in the experiment (Tab.5).

TABLE 4

Concentration of free amino acids in surface water samples from Stampenbäcken. One 1000 ml sample was analyzed each time.
A: upstream (Ravinen); B: middle section (Östarp); C: downstream (Hemmestorp).

ug/l	Date	A	B	C	A	B	C	A	B	C	A	B	C
		3.2	3.2	3.2	9.3	9.3	9.3	22.4	22.4	22.4	22.5	22.5	22.5
ala		102.5	65.9	25.8	21.8	10.3	2.1	23.6	18.6	2.8	4.3	7.4	3.5
gly		56.8	37.1	40.4	27.2	14.9	10.8	10.8	0.3	1.4	6.5	4.3	0.6
val		40.4	28.7	25.3	30.6	10.0	33.9	14.9	6.7	3.9	5.4	3.0	4.1
thr		10.4	7.5	6.4	10.8	8.4	9.2	5.9	8.2	7.6	3.7	0.8	5.7
ser		81.1	47.8	22.5	75.0	61.4	24.8	63.4	40.4	73.3	10.5	16.4	5.2
leu		5.7	3.9	3.3	12.7	7.3	7.6	0.8	0.6	0.7	0.5	1.7	1.0
pro		35.9	86.3	44.7	19.6	8.2	8.3	8.3	4.8	2.6	3.5	2.8	2.3
hypro		13.8	6.9	7.3	8.1	3.5	5.4	5.0	3.0	1.8	2.4	1.5	0.9
met		73.5	68.1	44.6	54.0	25.6	85.7	8.9	53.1	16.9	7.6	8.2	7.8
asp		25.7	18.6	7.5	10.4	4.3	7.3	1.3	3.1	0.8	0.9	1.6	0.4
phe		28.1	43.2	16.4	37.8	32.5	18.6	43.5	20.4	15.3	25.7	5.1	8.3
glu		8.9	13.6	0.5	1.0	0.4	1.3	0.1	0.2	0.2	0.2	0.2	0.3
tyr		4.7	8.0	6.2	2.2	1.7	1.5	6.3	0.4	1.8	2.3	1.3	1.4
lys		14.3	6.9	18.2	4.8	3.0	6.4	1.8	2.8	5.7	1.0	2.6	3.9
arg		53.9	36.7	30.5	75.7	22.5	18.4	26.7	7.1	3.9	18.3	23.6	4.8
his		51.3	24.8	29.3	14.9	8.3	38.1	1.6	5.2	10.1	4.6	8.0	3.4
try		5.7	8.5	7.9	6.4	3.2	3.8	3.1	2.4	2.3	3.5	2.2	1.5

Date	4.7			5.8			15.9			17.11		
ala	6.6	23.3	22.2	5.5	20.5	7.3	7.4	24.6	35.1	71.2	18.5	49.2
gly	0.1	11.2	18.0	0.7	10.4	7.2	11.2	42.1	38.5	13.8	11.2	7.8
val	0.6	8.2	19.8	1.9	20.2	16.2	6.6	39.2	35.6	36.8	41.7	28.1
thr	2.4	5.8	14.8	2.2	0.9	3.1	0.5	1.7	6.6	8.3	5.9	7.7
ser	51.3	66.4	6.7	0.5	28.0	4.2	4.9	93.6	11.1	92.1	64.3	46.2
leu	0.7	10.4	0.6	0.5	2.6	30.5	1.8	8.5	10.5	8.1	10.0	6.1
pro	3.6	11.6	2.1	4.7	18.4	12.1	2.7	21.0	9.0	50.8	76.9	13.5
hypro	0.2	1.4	0.3	0.1	0.3	0.1	0.5	0.5	8.6	5.8	4.4	8.9
met	13.2	58.9	18.7	11.8	40.7	34.5	8.9	80.4	40.6	45.2	14.1	18.6
asp	1.9	2.2	5.6	1.5	0.4	1.3	1.7	40.3	14.6	17.2	15.9	23.8
phe	8.0	10.1	6.4	4.3	2.5	6.0	2.1	12.7	7.3	21.2	18.6	25.4
glu	0.1	0.2	0.2	0.3	0.2	0.2	0.1	6.8	1.2	5.1	0.1	3.0
tyr	1.0	1.2	1.8	0.7	1.8	1.3	3.5	7.4	2.9	2.3	5.1	3.4
lys	2.4	3.0	0.6	0.8	4.2	3.4	0.9	13.6	8.4	5.7	8.2	9.4
arg	8.0	25.3	3.5	7.6	13.2	4.0	6.9	4.1	2.6	14.8	8.7	6.4
his	4.4	7.5	2.7	4.0	3.1	3.0	3.4	0.8	1.6	76.0	18.2	10.8
try	3.8	10.2	4.2	0.7	1.0	1.0	1.2	2.3	1.4	17.5	11.3	14.6

LENGTH OF FUNGAL MYCELIUM

Was the rapid decomposition of leaf proteins and subsequent absorption of free amino acids due to attached fungi, and was the difference in amino acid metabolism on alder and beech leaves due

¹ The numbers in Tab.2 are based on exudation from approximately 300 g leaves, immersed in 300 ml water.

TABLE 5

Concentration of free amino acids ($\text{mg}\cdot\text{l}^{-1}$) in a leaf drift water.
Mean of five analyses in the period November-February.

ala	8.5	asp	2.6
gly	3.0	phe	3.0
val	2.3	glu	2.6
thr	6.7	tyr	3.7
ser	1.6	lys	5.1
leu	4.3	arg	4.4
pro	3.9	his	3.9
hypro	1.5	try	3.2
met	0.9		

to structural differences in the fungal communities? Apparently, the length of fungal mycelium increased rapidly, and the increase was significant on both kinds of leaves (Tab.6). Mycelial length was significantly higher on alder leaves than on beech leaves, but the growth rate was essentially the same (analysis of covariance, $F=1.12$, $p < 0.5$). The mycelial length was larger on alder leaves than on beech leaves also in presence of Gammarus pulex and grazing did not significantly reduce mycelial length.

The fungi recovered rapidly from the HgCl_2 -treatment, the only apparent effect being a delay of the amino acid utilization. Treatment of the leaves with lincomycin and erythromycin seemed to be effective, since no bacterial colonies were detected in leaf suspensions in the method used. The pattern for amino acids build-up in water remained essentially the same, indicating that bacteria did not effectively compete with fungi for the free amino acids in the experiment.

TABLE 6

Length of fungal mycelium (m/g wet weight) in alder and beech leaves during incubation in water. The results are statistically tested by one-way ANOVA.

Incubation days	0	4	7	21	56	F	p
alder	963.2	1143.7	1316.1	1637.4	1875.4	5.62	<0.025
beech	583.7	641.5	808.0	1281.6	1504.8	4.21	<0.025
alder+amphipod	952.8	1127.1	1274.6	1635.6	1748.4	5.18	<0.025

SPECIES COMPOSITION

The number of fungal species was much lower on beech leaves than on alder leaves (Tab.7). Only three out of twelve species were identified on beech leaves; one of these species, Tricladium splendens, was not found on alder leaves. The other two species on beech leaves, Clavariopsis aquatica and Anguillospora longissima were also abundant on alder leaves. The species identified certainly are those that metabolized the experimental leaves, but the time of exposure of leaves in stream water was probably too short to establish an equilibrium between immigration and extinction of species (Bärlocher 1980). However, referring to the conclusion by Sanders and Anderson (1979), the identified species may be the stable component of the guild that may sporadically be enriched with others.

TABLE 7

Frequency of identification of aquatic hyphomycetes on alder and beech leaves, expressed as % of total number of conidia counted on each leaf sort.

	alder	beech
Tetracladium marchalianum (deWild)	21	0
Clavariopsis aquatica (deWild)	15	30
Clavatospora longibrachiata (Ingold)	1	0
Lemonniera aquatica (deWild)	13	0
Flagellospora curvula (Ingold)	2	0
Anguillospora longissima (deWild)	16	37
Alatospora acuminata (Ingold)	9	0
Tetracladium setigerum (Ingold)	3	0
Margaritospora aquatica (Ingold)	4	0
Tricladium splendens (Ingold)	0	33
Articulospora tetracladia (Ingold)	13	0
Clavatospora stellatus (Ingold & Cox)	3	0

PREFERENCE FOR PROTEIN BOUND OR FREE AMINO ACIDS?

When leaves at different stages of decay were incubated in water along with different concentrations of free amino acids, the same amount of free amino acids was absorbed during each of the five sampling periods (coefficient of variation for total amino acid concentration less than 20 %) in each of the 4 leaf decay stages x 4 leachate concentration experiments. The mean for each set of

TABLE 8

The average amounts (mg/g leaf tissue) of protein amino acids (I) and free amino acids (II) absorbed during incubation in water of alder leaves.

Amino acids in leaves at start of incubation, mg/g	14.18		6.12		5.35		2.68	
Free amino acids in water, mg/g	I	II	I	II	I	II	I	II
0.5	7.4	0.1	3.5	0.2	2.5	0.2	1.3	0.2
1.0	6.5	0.3	3.0	0.3	2.8	0.5	1.0	0.5
2.0	6.0	0.4	2.7	0.8	2.0	1.3	0.8	0.7
4.0	5.7	1.4	2.4	1.9	2.0	1.8	0.5	2.1

experiment is given in Tab.8. To calculate the amount of leaf amino acids that was hydrolyzed and absorbed during each experiment, the concentration in the leaves at the end of each incubation was subtracted from the concentration at the start. A correction was made for the amount of leaf amino acids that was hydrolyzed but subsequently released into the water. In the experiment it varied between 26 and 48 %, regardless of the concentration of free amino acids or protein, and an average of 35 % was used for correction. The average amount of leaf amino acids absorbed decreased as the concentration of free amino acids in the water decreased, for all four stages of leaf decay, while the amount of free amino acids absorbed increased (Tab.8). When the ratio of absorbed free amino acids to absorbed protein amino acids was plotted versus the ratio of available free amino acids to protein amino acids, it was found that the proportion absorbed did not correspond linearly to the proportion available (Fig.4). Less quantities of free amino acids than expected were absorbed when the relative availability was less than 30 %, and more than expected was absorbed when the relative availability increased above 40 %.

DISCUSSION

The experimental evidence that an exponential model adequately describes the decomposition of leaf proteins has been emphasized by Kaushik and Hynes (1968). They found that the concentration

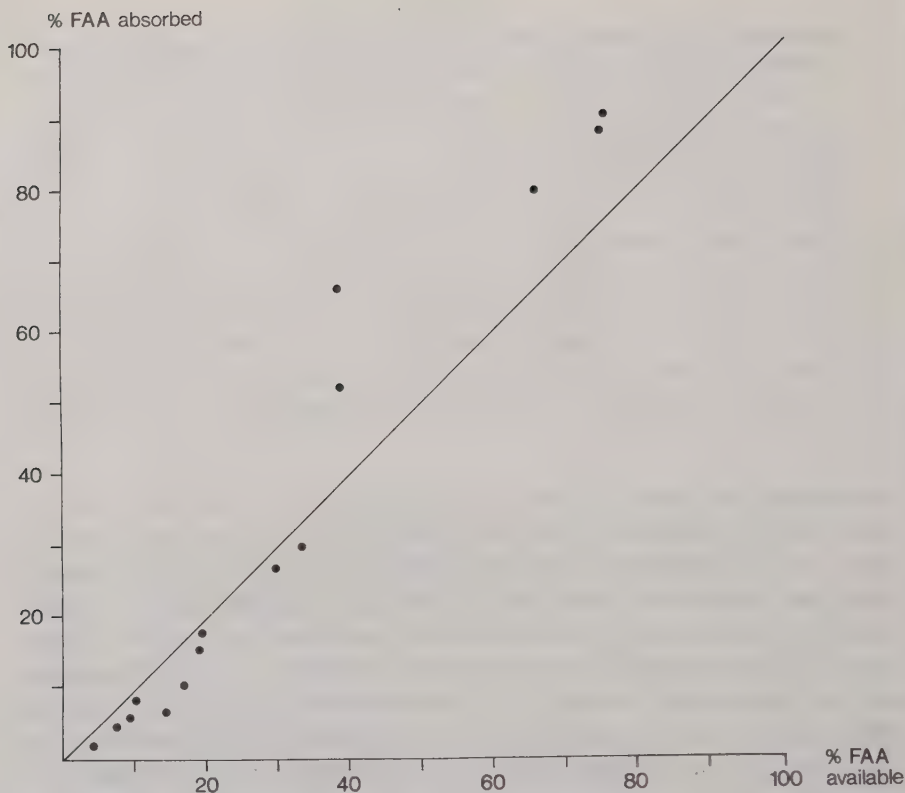


Fig.4. Data for the ratio of available free amino acids to available protein amino acids plotted versus the ratio of absorbed free amino acids to absorbed protein amino acids. Data from Tab.8.

of insoluble proteins approached a constant level after 35 days in water. However, there are conflicting evidence as to the depletion of the organic nitrogen pool in leaves. Anderson (1973) and Iversen (1973) showed that total N increased in beech leaves during decomposition. An increase of nitrogen has also been found in leaves of other species (Howard and Howard 1973, Triska et al. 1975, Suberkropp et al. 1976). The conflicting results may be due to differences in leaching and microbial activity on the leaves. It has been anticipated that leaching explains the very rapid initial loss of low molecular weight compounds from leaves (Nykvist 1963, Kaushik and Hynes 1971, Petersen and Cummins 1974), and the subsequent increase of nitrogen may be a consequence of selective immobilization of N by the increasing microbial biomass.

Although a strong influence of leaching on the results in this experiment was virtually eliminated by pre-leaching of the leaves, the evidence for microbial processing of leaf proteins remained indirect, since the non-disruptive sterilization method was not efficient for the full length of the experiment. However, microbial decomposition, which was traced both by amino acid analysis (Tabs.2,3) and growth of fungal mycelium (Tab.6), was predominating from the first day of incubation, and a rapid drop and subsequent build-up of protein nitrogen was not expected. Also if the content of nitrogen would not be comparable in the references mentioned because of different rates of leaching and hydrolysis of other elements than nitrogen, the fact that only the concentration of protein amino acids and not total N was analyzed in this experiment is important. Assuming that the percentage nitrogen in less soluble fractions, such as humic acids and fulvic acids, was between 1 and 5 and that these fractions represented only a minor part of the leaf tissue, say 25 %, their nitrogen content may have been a large part of total N. If so, decomposition of total N and protein would certainly not show the same pattern, and estimates of the available nitrogen pool would be different. However, also a certain fraction of protein was less hydrolyzable for microorganisms (Tab.1). Provided about 1.5 mg/g of amino acids was less available, about 12 mg/g in alder and 2.5 mg/g in beech leaves was readily available for microbial metabolism. If the available amino acids were a limiting nutrient and energy source for fungi, the quality of the leaves may impose a selective advantage, and it would be more advantageous to exploit alder leaves than beech leaves.

Also if the estimated benefit of colonizing alder leaves with respect to amino acids was enhanced by less amounts of unpalatable secondary plant substances (cf. Suberkropp and Klug 1976, King and Heath 1967), the heavy colonization is noteworthy, since alder leaves were rapidly consumed by macroinvertebrates in the natural water. It may be that fungal species through natural selection have developed different strategies on different leaves. Adding a new factor, feeding physiology, to the scheme given by Pianka (1970), resolves another correlate ("simplicity/complexity") of the selective forces acting on leaf decomposition. Fungi on alder leaves should be opportunistic, favoring competitive growth and dispersal and absorption of dissolved nutrients. On beech leaves selection should favor equilibrium species with effi-

cient extracellular enzymatic machinery, and anti-herbivore defence mechanisms.

Regardless of the functional implication, the fungal community on alder and beech leaves was different (Tab.7) and several species were absent on beech leaves. *Tricladium splendens* seemed to be a key species to understand differences in microbial strategies on the leaves (for further data, see Bengtsson (subm.)).

The more rapid and complete absorption of free amino acids by alder leaf fungi also supported the suggested strategy scheme (Fig.3). The alder leaf community species responded faster to the increased concentration of free amino acids in the environment and also reduced the amino acid concentration to a relatively lower value. How efficient were amino acids absorbed by fungi? The data showed that fungi on alder leaves at a certain supply of free amino acids started to absorb a higher proportion than what would be expected if the absorption was directly proportional to the concentration available (Tab.8). This superproportional utilization behaviour, which has been termed switching, has been described for vertebrates (Murdoch 1969, Murdoch et al. 1975) and for aquatic invertebrates (Lawton et al. 1974). The switching probably reflects an optimal "choice"; the transport into the cells of readily available free amino acids gives a better yield of energy and nitrogen than the enzymatic decomposition of leaf proteins, although the concentration of the latter is several orders of magnitude greater. Switching also requires an active transport system, and it has been shown that amino acids accumulate against a concentration gradient in fungal mycelia (Bent and Morton 1964b).

The experimental system was self-regulatory to the extent that at least part of the community now and then had to switch back to enzymatic hydrolysis of leaf proteins to maintain the level of free amino acids found in the water. During periods with high concentrations of free amino acids in the water, fungi are thus more likely to use leaves as a pure substrate. The situation in a natural water is certainly more complicated to describe, especially as it is difficult to sample the water that supplies nutrients to the fungi. The seasonal variations of amino acid concentrations in surface water may be important for the switching behaviour. The variation is basically related to two sources, leaf fall and soil surface runoff, the peak of which also occurs in fall and winter due to heavy precipitation. Both sources may contribute

all of the amino acids analyzed, although some of them are likely more abundant in surface runoff. Serine, which is predominating in marine waters (Clark et al. 1972, Schell 1974), and methionine, which is abundant in surface stream water (Tab.4), were much less abundant in leaf leachate (Tab.2) and in the leaf drift (Tab.5).

The build-up of amino acids in the water of the experimental bottles apparently reflected processes that lead to the accumulation of free amino acids in a leaf drift, although the concentrations of amino acids were higher in the leaf drift than ever reached in the experimental bottles. Obviously, analysis of amino acids in surface stream water cannot be used to predict the nutritional contribution of these compounds to microbial metabolism in sediment environments. The differences in concentration between the surface stream water and the leaf drift water may be of the same ecological importance as the differences found in sea water between open water and pore water in interstitial biotopes (Dawson and Prichard 1978, Henrichs and Farrington 1979, Jørgensen 1979).

The observations described predict that aquatic hyphomycetes exploiting detritus in a stream switch to dissolved amino acids at a certain relative concentration. In northern Europe this should occur during fall and winter, when the most available protein bound amino acids are metabolized. Switching should be frequent when dense leaf drifts are formed in small calm coves with almost stagnant water, where the free amino acid concentration is even larger than in the experiment described in this paper. Enrichment of a stream with dissolved organic nitrogen from other sources may further decrease the decomposition rate of detritus by the described fungal behaviour.

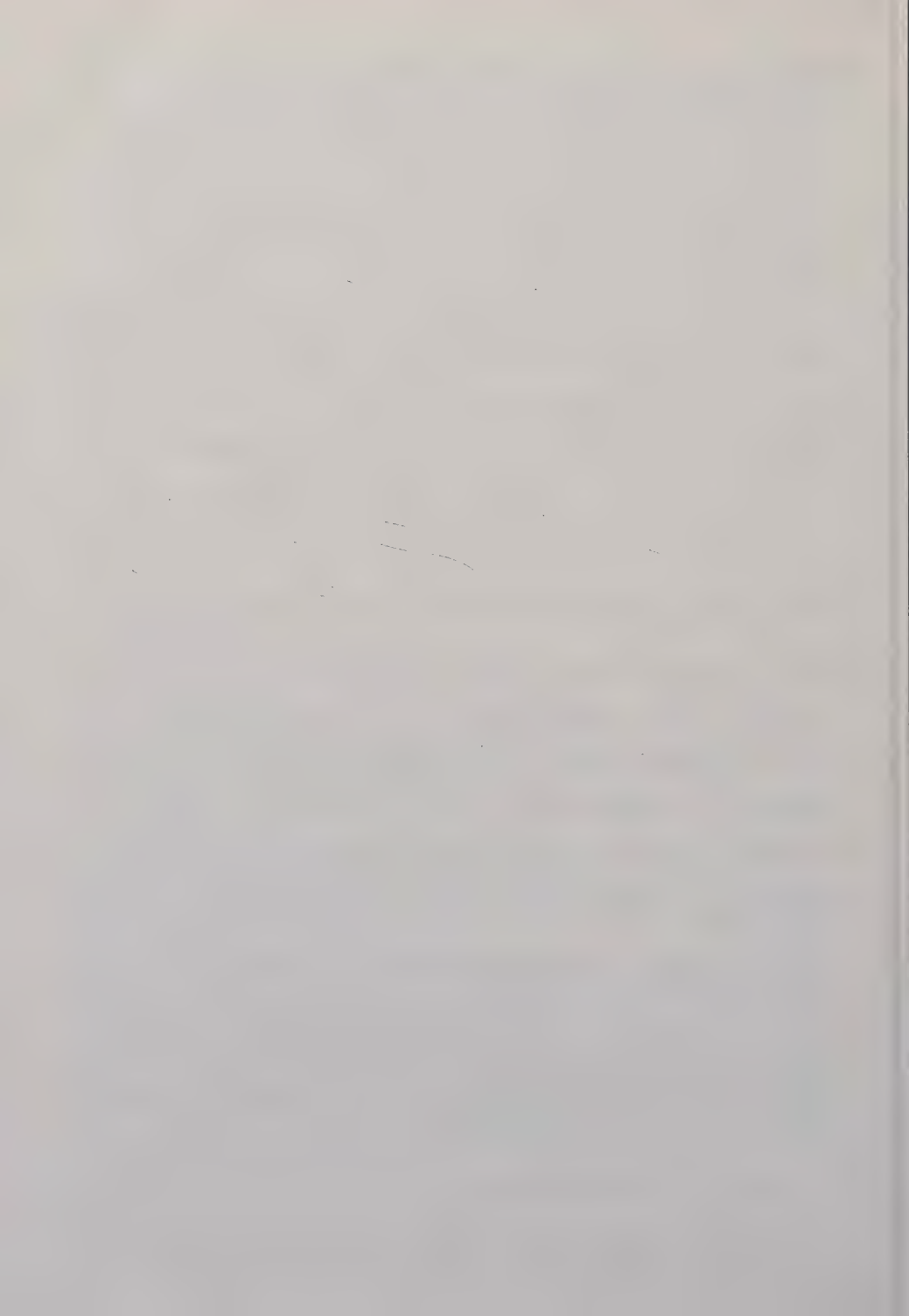
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HABITAT SELECTION IN TWO SPECIES OF AQUATIC HYPHOMYCETES

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ABSTRACT

Two species of aquatic hyphomycetes, Tetracladium marchalianum and Tricladium splendens, were isolated from decaying leaves in a stream. T. marchalianum was abundant on alder leaves but absent on beech leaves, which were dominated by T. splendens. It was hypothesized that differences in some chemical key factors in the leaves would account for differences in the distribution of the two species. In the experiment designed to test the hypothesis, combinations of sterilized leaves and isolated fungi were used. Differences in growth of FDA active mycelium were related to differences in leaf weight loss; T. splendens decomposed beech leaves and T. marchalianum alder leaves. Extracellular protease activity corresponded with these trends but there was no detectable protein loss in the leaves. Both fungi showed a nitrogen demand and hydrolysis of leaf proteins was complemented with absorption of free amino acids, ammonium, and nitrate. High concentrations of free amino acids modified the pattern for habitat selection so that T. splendens grew substantially on alder leaves and T. marchalianum colonized beech leaves. No protease activity was, however, found from T. marchalianum on beech leaves and it is concluded that a more general metabolic inhibition prevents extensive growth of this species on beech leaves. The low natural abundance of T. splendens on alder leaves, where it may grow well, may be a consequence of a specific proteinase inhibition and competition from other species.

INTRODUCTION

Decay of autumn-shed leaves in a stream is promoted by associated microorganisms, which are numerous on most leaves (Kaushik and Hynes 1971, Bärlocher and Kendrick 1974, Suberkropp and Klug 1976). It is generally assumed that these microorganisms have recourse to enzymes for decomposition of most leaf substances, but evidence indicate that also dissolved organic matter in the water is absorbed (Bärlocher and Kendrick 1974, Bengtsson subm.).

Microbial preference for dissolved organic matter at high concentrations may cause a temporary decline in leaf decay, but the low decay rate of certain leaves, e.g. beech leaves, more likely depends on the leaves' chemical nature. Beech leaves have few species of aquatic hyphomycetes, the group of microorganisms that tend to dominate early stages of leaf decay (Kaushik and Hynes 1971, Suberkropp and Klug 1976), compared to e.g. alder leaves (Bengtsson subm.), and it has been anticipated that this is because strong polyphenol bindings prevent enzymatic hydrolysis of leaf proteins (Suberkropp and Klug 1976).

The chemical composition of leaves would thus select for guilds of fungi with special physiological adaptations on different leaves, and with this in mind some laboratory experiments were performed to test a number of hypotheses. Two species of aquatic fungi were isolated from autumn-shed leaves and used in the expe-

riments. One of them, Tricladium splendens, was predominant on beech leaves, and the other, Tetracladium marchalianum, was abundant on alder leaves.

The two species were used in combination with the two kinds of leaves to test following hypotheses: a) aquatic hyphomycetes are habitat selected with more efficient metabolism and decomposition on certain kinds of leaves, b) fungi that dominate on slowly decaying leaves, have a high mycelial growth rate on both slowly decaying and rapidly decaying leaves, if not limited by competition or selective predation. Abundant species on rapidly decaying leaves have a high mycelial growth rate on these but a lower growth rate on slowly decaying leaves, c) extracellular protease activity is higher for fungi dominating on slowly decaying leaves, d) free amino acids are more efficiently absorbed from the water by fungi that dominate rapidly decaying leaves, e) free amino acids is a more eligible nitrogen source for aquatic fungi than nitrate or ammonium.

MATERIAL AND METHODS

Alder (Alnus glutinosa L.) and beech (Fagus sylvatica L.) leaves were collected from two 4 m² nylon nets that were stretched above a stream bordered by trees. Leaves were enclosed in 2 mm mesh nylon bags and left in the stream for two days to ensure proper conditioning. The bags were brought to the laboratory and the leaves rinsed in cold tap water to remove extraneous sediment and invertebrates. The leaves were moved to plastic jars, leached in running tap water for 2 days, and dried in an oven at 60° C for 2 days. Leaves were weighed and then placed in 250 ml Erlenmeyer flasks, one leaf in each flask, stoppered with a cotton plug. The leaves were autoclaved at 1 atö for 15 min and 100 ml distilled autoclaved water added to each flask. The flasks were then subject to individual treatments (Fig. 1).

ISOLATION OF FUNGI

Fungi were isolated from decaying leaves in the stream according to Webster (1959) and Bärlocher and Kendrick (1974). Briefly, some leaves of each species were kept in distilled water in Petri dishes in a culture chamber at 10° C for 1-3 days. When sporula-

tion was induced spores were picked up by a Pasteur pipett and planted on 2 % malt agar to which chlortetracycline was added. After one week's incubation at 20° C strips of colonies were transferred to tubes with sterilized distilled water.

Some leaves were cut into 1 mm² pieces and plated on 2 % malt agar in Petri dishes. Colonies, which appeared after one week, were cut in strips and submerged in tubes with sterilized distilled water. Spores were inoculated on 2 % malt extract agar in Petri dishes and bacteria free isolates were established and maintained at 20° C until used for experiments. Spores to be used as inoculum in experiments were produced by submerging pieces of mycelium in 100 ml Erlenmeyer flasks with sterilized distilled water. Heary sporulation was usually induced after 3-4 days.

Tetracladium marchalianum was used in this work as a species that was exclusively found on alder leaves (Bengtsson subm.). Tricladium splendens was quite frequent on beech leaves but rarely found on alder leaves.

Glycine, glutamic acid and lysine were selected to represent a simple amino acid (glycine), a more complex compound that hyphomycetes have grown well on (glutamic acid, Thornton and Fox 1968) and a diamino acid (lysine) and added to some flasks (Fig. 1) at a concentration of 10 g/l. The flasks were incubated in circulating distilled water at 15° C (Heto refrigerator) and sampled after one and two weeks. The water in the flasks was provided with a gentle stream of filtered air (0.22 µm disposable Millipore filter) through a combined glass and plastic tubing connected to a compressed air tank.

CHEMICAL ANALYSES

Weight losses of leaves were calculated after drying leaves at 60° C for 2 days and weighing.

Protein content of leaves was calculated by weighing a subsample (20 mg) of a leaf, dried for 2 days in a desiccator, into a screw cap culture tube, adding 6M HCl and hydrolyzing at 110° C for 24 h. Residual leaf tissue was filtered on a 20 µm Whatman filter and the filtrate analyzed for protein amino acids using capillary gas chromatography and electron capture detection (Bengtsson and Odham 1979, Bengtsson et al. 1981). This method is sensitive and less than 1 pg of amino acids can be detected.

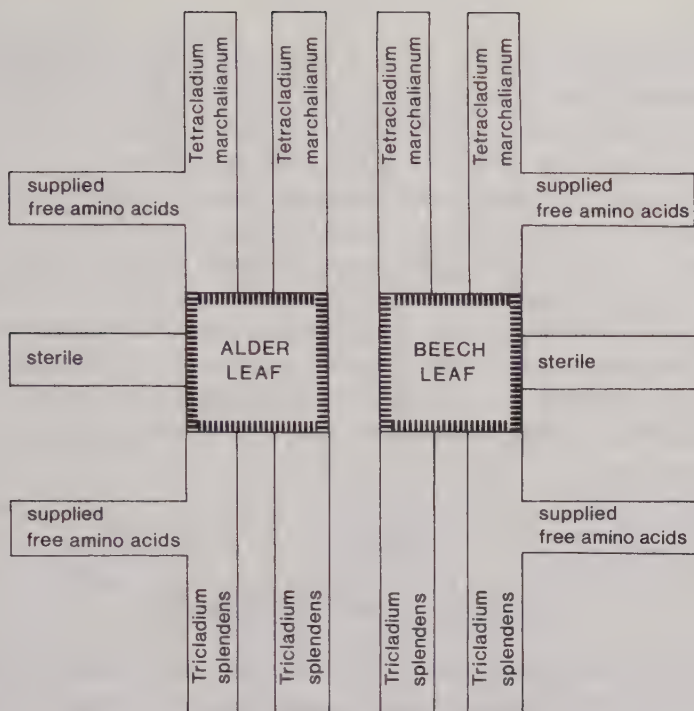


Fig. 1. Design of experiment.

Flasks were shaken and the water vacuum filtered (0.45 μm). 15 ml was used for free amino acid analyses, 25 ml was used for nitrate and ammonium analyses, and 6 ml was used for measurement of protease activity. Nitrate and ammonium were analyzed immediately by ion selective electrodes on an Orion 901 using a 10 ml sample for each ion. Protease activity of the filtrate was measured using an insoluble chromogenic protein, azocoll. 30 mg azocoll was added to the filtrate and the suspension swirled. Samples were taken after 10 min (to correct for background colour) and 300 min incubation at 25 $^{\circ}$ C. The absorbance of the suspension was determined at 520 nm.

Samples of leaf tissue from 0.2 - 1.0 g each were homogenized in 100 ml phosphate buffer (60 mM, pH 7.5) for 1.5 min by a Sorvall^R Omni-mixer at half speed. The suspension was diluted 10 times and fluorescein diacetate (FDA, 10 µg/ml) was added. The hyphae were stained for 3 min and then collected on a 22 mm membrane filter (0.8 µm Millipore). The filter was placed on a drop of non-fluorescent immersion oil on a glass slide, another drop was added on top of the filter, which was covered with a cover glass. Mycelial length was measured with the inter-section method (Söderström 1977) in a Reichert Zetopan microscope equipped with a Binolux light source. Subsamples for control of bacterial contamination were taken from leaves, homogenized and spread onto plates of leaf extract agar.

RESULTS

IS LEAF DECAY SPECIES DEPENDENT?

Inoculation of sterilized leaves with fungal spores significantly influenced the weight loss of the leaves (Tab. 1). Decay of beech leaves was promoted by T. splendens, which required high concentrations of free amino acids in the water to decompose alder leaves. T. marchalianum reduced the decay of beech leaves, and did not even in presence of excess nitrogen initiate a detectable decomposition of beech leaves. On the other hand, T. marcha-

TABLE 1

Weight loss (% , 3 replicates) of alder leaves and beech leaves incubated at 15°C. t-test made on differences between sterilized leaves and inoculated leaves.

Treatment	Beech leaves		Alder leaves	
	1w	2w	1w	2w
sterilized leaves	4.77	8.53	5.21	8.48
inoc. <u>T. splendens</u>	8.07***	14.68***	4.26***	7.80***
inoc. <u>T. splendens</u> + free amino acids	3.36**	7.84**	7.30***	9.13**
inoc. <u>T. marchalianum</u>	5.20*	5.45***	5.43	10.25***
inoc. <u>T. marchalianum</u> + free amino acids	5.12*	7.83**	6.18**	10.04**

TABLE 2

Total concentration (mg/g wet weight) of protein amino acids in hydrolyzed alder and beech leaves. Calculations are based on the concentration of individual amino acids. Mean (n=3) and SD is shown as well as significance of difference (t-test) between sterilized leaves and inoculated leaves.

Treatment	Beech leaves		Alder leaves	
	1w	2w	1w	2w
sterilized leaves	7.1±0.6	4.5±1.2	26.6±1.6	12.1±2.4
inoc. <u>T. splendens</u>	11.3±1.0***	1.7±0.3***	28.2±2.1	15.7±1.5
inoc. <u>T. splendens</u> + free amino acids	12.8±1.4***	15.3±2.2***	31.7±2.8**	22.3±2.7***
inoc. <u>T. marchalianum</u>	36.4±2.8***	30.5±1.7***	30.3±3.4*	9.4±0.6**
inoc. <u>T. marchalianum</u> + free amino acids	28.3±1.9***	25.1±2.0***	91.4±8.7***	62.6±3.1***

lianium increased the decay of alder leaves significantly, regardless of the concentration of free amino acids in the water. The decay of the leaves was thus dependent on the specific fungi colonizing the leaves but also on the concentration of free amino acids in the water.

DO DIFFERENT FUNGI UTILIZE LEAF PROTEIN TO THE SAME DEGREE?

Presence of any of the fungi on any of the leaves significantly increased the concentration of protein amino acids of the leaves (Tab. 2). T. marchalianum induced the highest protein concentration in beech leaves, while T. splendens, though increasing the protein content *per se*, reduced the concentration of two amino acids, threonine and serine, to a lower level than in sterilized leaves. The increase of protein in alder leaves was especially great in presence of T. marchalianum and excess amounts of free amino acids.

Any true increase of protein in leaves may be obscured by the fact that other groups of compounds, such as carbohydrates, may be leached or utilized more rapidly than amino acids, thus generating a certain weight loss, which tends to increase the relative proportion of protein in leaves. The relative protein changes in leaves due to different treatments can be compared with the relative weight losses to reveal any real transformation of protein amino acids. Some differences become obvious by comparing Tab. 1 and Tab. 2.

The significant protein increase in beech leaves in presence of T. splendens may be an artifact of the great weight loss; thus the data are not in support of any real protein increase. The same conclusion can be extended to the relation between T. splendens and alder leaves in presence of excess amounts of free amino acids. On the other hand, T. marchalianum evidently increased the protein content of beech leaves, since the weight loss was less than for sterilized leaves. Although there was a greater weight loss in alder leaves inoculated with T. marchalianum and enriched with free amino acids, the loss seemed to be too small to explain the very high protein increase. It thus seems reasonable to conclude that the presence of T. marchalianum on both alder and beech leaves may increase the protein content of the leaves, while T. splendens may not. Whether the protein increased because of dense growth of protein rich mycelium or actual transport of amino acids, peptides or other dissolved nitrogen compounds into the leaf tissue was not demonstrated by this experiment.

DO FUNGI WITH HIGH ABUNDANCE AT ALDER LEAVES ABSORB FREE AMINO ACIDS MORE EFFICIENTLY?

In a river, autumn-shed alder leaves may be decomposed within three weeks, mainly because of fragmentation by invertebrates, while beech leaves will remain in leaf packs for months (unpubl. observation). If these differences in habitat duration are related to differences in utilization of free and protein bound amino acids by fungi, then T. marchalianum should be more efficient on absorbing FAA than T. splendens, which has a more predictable protein source in beech leaves. Although it was not possible to quantify the absorption of FAA in this experiment, a comparison with sterile leaves showed that T. marchalianum reduced the concentration of most free amino acids in the water around both kinds of leaves after two weeks incubation (Tab. 3). After one week of incubation leaching still exceeded absorption and also after two weeks for some amino acids leached from beech leaves. T. splendens absorbed a much less proportion of leached amino acids, and absorption exceeded leaching only for some few amino acids. Generally, the pool of free amino acids was reduced to its lowest level by T. marchalianum.

TABLE 3

Concentration of free amino acids ($\mu\text{g/g}$ wet weight of leaves) in water.
Means are given for $n = 3$, * = $\times 10^3$, ** = 10^6 , FAA = free amino acids.

Means are given for n = 37																								
Alder leaves					Beech leaves																			
1w					2w					1w					2w									
F. marchalianum					F. splendens					F. marchalianum + FAA					F. splendens + FAA					F. splendens + FAA				
sterile					sterile					sterile					sterile					sterile				
ala	63	32	57	26	98	116	52	132	74	381	38	41	69	36	63	44	45	88	53	41				
gly	43	31	86	81*	65*	54	22	75	3*	18*	30	58	33	4*	714	35	14	85	1.5*	316				
val	66	85	75	63	75	114	94	106	35	12	64	33	73	58	22	61	37	264	43	47				
thr	101	451	215	105	84	142	53	168	110	156	3	16	275	22	2	21	15	418	24	5				
ser	41	23	83	71	36	74	73	154	9	28	95	83	121	39	96	114	110	128	14	59				
leu	38	178	25	3	14	79	42	87	2	1	24	51	201	18	176	31	44	115	3	56				
pro	14	13	53	13	1	16	69	6	6	23	6	10	48	14	2	10	16	13	17	5				
met	16	50	23	50	9	42	125	51	16	40	14	159	20	6	16	23	12	79	11	23				
asp	7	35	14	19	16	10	77	5	3	5	5	5	49	13	28	24	1	82	20	36				
phe	13	81	38	65	15	25	23	43	8	16	8	21	184	29	235	19	36	95	8	114				
glu	3	4	61	952	3.6*	28	5	102	518	1.3*	43	18	152	1.7*	815	62	34	112	873	410				
tyr	62	80	75	63	50	103	25	98	54	88	21	23	87	115	61	27	30	62	73	18				
lys	36	321	51	932*	2.0**	43	115	73	861*	1586*	45	18	742	83	5.5*	49	46	296	64*	2.6*				
arg	19	62	26	46	32	35	75	86	39	41	81	78	200	53	61	96	103	132	70	24				
his	44	29	18	24	59	73	91	10	3	18	17	14	53	20	8	19	15	42	11	17				
try	27	51	25	6	39	31	235	56	11	35	6	19	56	18	10	16	20	75	9	6				

Addition of glycine, lysine, and glutamic acid had a dramatic effect on the free amino acid absorption as a whole, especially for T. splendens. Absorption exceeded leaching for several amino acids even for this species. Glycine and glutamic acid was rapidly metabolised by both fungi, more efficiently by T. marchalianum on alder leaves and by T. splendens on beech leaves, while lysine was much less readily absorbed.

IS ABSORPTION OF NITRATE/AMMONIUM SUBORDINATED

ABSORPTION OF FAA?

A difference in the metabolism of free amino acids and nitrate/ammonium appears if Tab. 3 is compared with Tab. 4. While a great proportion of leached amino acids were utilized in presence of the excess amino acid source, excess nitrate and ammonia was produced by both species of fungi and on both kinds of leaves, at least after two weeks incubation. It seems reasonable to assume that nitrate and ammonia were end products of the amino acid metabolism, and that the fungi rather used free amino acids as an energy source than as a nitrogen source, when in surplus. With only one exception, T. splendens exuded the largest amounts of inorganic nitrogen. There was a lag time in exudation so that after one week more ammonia was found from cultures of T. marchalianum.

TABLE 4

Concentration of nitrate and ammonium ($\mu\text{M/g}$ leaf) in water.
Means for $n = 3$ are given.

Treatment	Beech leaves				Alder leaves			
	NH_4^+		NO_3^-		NH_4^+		NO_3^-	
	1w	2w	1w	2w	1w	2w	1w	2w
sterile	38.1	41.7	2188	5669	43.7	88.2	616	2881
inoc. <u>T. splendens</u>	33.4	39.0	2265	1524	42.9	7.1	676	1700
inoc. <u>T. marchalianum</u>	72.2	62.5	1670	1528	27.6	24.0	842	2184
inoc. <u>T. splendens</u> + FAA	1043	1162	1964	171606	176	1194	308	3082
inoc. <u>T. marchalianum</u> + FAA	684	789	1733	13053	735	722	2395	7767
inoc. <u>T. marchalianum</u>		4444	4444	4444	4444			

TABLE 5

Protease activity (A_{520} /g leaf) in water. Mean for $n = 3$ is given.
nd = not detected.

Treatment	Beech leaves		Alder leaves	
	1w	2w	1w	2w
sterile	nd	nd	nd	nd
inoc. <u>T. splendens</u>	0.52	1.35	nd	0.19
inoc. <u>T. marchalianum</u>	nd	nd	0.37	0.42
inoc. <u>T. splendens</u> + FAA	0.15	0.18	0.11	0.23
inoc. <u>T. marchalianum</u> + FAA	nd	nd	nd	nd

When the water was not supplied with the external free amino acid source, leached ammonia was absorbed by both fungi on both kinds of leaves, irrespective of incubation time. On beech leaves ammonia was most rapidly used by T. splendens, while T. marchalianum most rapidly absorbed ammonia from alder leaves. However, after two weeks incubation, T. splendens had removed most of the leached ammonia from alder leaves. Also nitrate, generally, was more efficiently absorbed by T. splendens on both kinds of leaves, so the minor absorption of low concentrations of free amino acids by this species seemed to be compensated to some extent by absorption of nitrate and ammonium. Also T. marchalianum absorbed nitrate and ammonium most of the time, so this species, so far, seemed to be a decided absorber.

DO FUNGI WITH HIGH ABUNDANCE ON BEECH LEAVES PRODUCE

MORE EXTRACELLULAR PROTEASE?

Release of extracellular protease was dependent on a specific combination of leaves and fungi. Highest protease activity was found in waters with beech leaves inoculated with T. splendens (Tab. 5), while T. marchalianum on alder leaves produced slightly less protease activity. Extracellular protease production was decreased by T. splendens on beech leaves in presence of excess glycine, lysine and glutamic acid, while a surplus of free amino acids in the water seemed to be a necessary condition for T. splendens to produce proteases on alder leaves.

No extracellular proteases were found from T. marchalianum on beech leaves, indicating none or a very low protease activity, and excess amounts of glycine, lysine and glutamic acid in the water inhibited the protease activity on alder leaves.

The overall pattern for protease activity coincided well with the weight loss of leaves (Tab. 1). The protease attack by T. splendens on beech leaves and by T. marchalianum on alder leaves caused a corresponding weight loss.

IS GROWTH OF FUNGI HABITAT SPECIFIC?

The mycelium of T. splendens was more developed on beech leaves than on alder leaves and grew faster than the mycelium of T. marchalianum, even on alder leaves (Tab. 6). Beech leaves seemed to be a very unfavourable growth substrate for T. marchalianum and addition of excess amounts of glycine, lysine and glutamic acid to the water increased the mycelial length 20 times; however, its mycelium was still shorter than that of T. splendens. Excess amounts of FAA also stimulated growth of T. splendens, nearly doubling mycelial length on beech leaves and increasing it 20 times on alder leaves. High concentrations of free amino acids thus seemed to be relatively more important for a growth increase in T. splendens on alder leaves than on beech leaves. Although excess amounts of FAA resulted in a substantial increase of mycelial length, a peak in "biolength" evidently was reached within one week. Between one and two weeks incubation, length of active mycelium declined to half of its maximum length on alder leaves for both fungi. No colonies of bacteria were found on any leaf.

DISCUSSION

If the distribution of fungi on different leaves is not dependent on chemical differences between the leaves, then at least one condition must be met, namely identical mycelial growth rates by the same species on different leaves in a single species environment. However, even if growth rates are different, it does not follow directly that chemical compounds in the leaves are utilized but the leaves simply used as a substrate while the fungi absorb compounds dissolved in the water. It then clearly needs to be asses-

TABLE 6

Length of FDA-active mycelium (m/g leaf) in leaves. Means of $n = 3$ are given.

Treatment	Beech leaves		Alder leaves	
	1w	2w	1w	2w
sterile	nd	nd	nd	nd
inoc. <u>T. splendens</u>	273	109	24	79
inoc. <u>T. marchalianum</u>	nd	4.4	nd	70
inoc. <u>T. splendens</u> + FAA	450	412	540	271
inoc. <u>T. marchalianum</u> + FAA	nd	86	70	22

sed whether the dissolved compounds are released from the leaves by leaching or by enzymatic attack by the fungi.

Data on mycelial growth of the two species on beech and alder leaves give no support for the hypothesis that differences in the chemical composition of the leaves are unimportant for habitat selection (Tab. 6). Mycelial length of T. marchalianum on beech leaves was less than one tenth of the mycelium on alder leaves after two weeks incubation but after addition of dissolved amino acids reached a higher peak than on alder leaves. It seems likely that a key growth factor for this species was amino acids, which remained unhydrolyzed in the beech leaf proteins. What inhibited protein utilization is unknown. At least, it was not a toxic compound since the species grew well in presence of dissolved amino acids. The rapid growth of T. splendens on alder leaves is interesting in the light of its low natural abundance (Bengtsson subm.) and the slow growth of T. marchalianum. It indicates that competition or selective predation might be relevant factors for habitat selection on alder leaves.

Is the difference in growth rate between the fungi related to differences in decay of the habitats? Indirect evidence for the contribution by microbial communities to the decay of autumn-shed leaves have been published (Bärlocher and Kendrick 1974, Suberkropp and Klug 1976) but data presented in Tab. 1 demonstrate a great variability in decomposition efficiency in fungi. The difference in mycelial length of T. marchalianum on beech and alder leaves corresponded to a similar difference in leaf decomposition (Tab. 1); only compounds in alder leaves were utilized to some

degree, indicating the species' discrimination between the different leaf tissues. Apparently, the presence of dissolved free amino acids increased the initial utilization of leaf compounds, especially on alder leaves. The metabolism of dissolved amino acids thus seemed to be related to the activity or efficiency of its enzymes. Also T. splendens discriminated between the two kinds of leaves and its superior growth on beech leaves was accompanied by an extensive leaf decay. As for T. marchalianum, presence of high concentrations of free amino acids modified the specific relation between T. splendens and beech leaves, so, in fact, alder leaves were more decomposed than beech leaves.

But why did T. marchalianum grow so slowly on beech leaves, with no detectable protease activity, and why was T. splendens' protease activity so low on alder leaves? The most obvious explanation is that the metabolism of the fungi was inhibited. The inhibition of T. marchalianum seemed to be more like a specific protease inhibition than a more general metabolic inhibition, since the fungi grew and metabolized amino acid exudates on beech leaves. This metabolic pattern was basically true also for T. splendens on alder leaves, so the two species probably were sensitive to different proteinase inhibitors, most likely because their proteinases have a different chemical structure. As the proteinase activity of the two fungi varied inversely on the two kinds of leaves, the data did not support the hypothesis that protein amino acids were made unavailable to fungi by complexing with leaf phenols (King and Heath 1967, Suberkropp and Klug 1976). However, this does not exclude phenols as a possible source for the metabolic inhibition, since many phenolic acids are known as strong fungicides.

Did excess amounts of free amino acids in leaf surroundings modify the pattern for habitat selection so that T. splendens grew as good or better on alder leaves compared to beech leaves, reducing the leaf weight significantly, and T. marchalianum inhabited beech leaves with similar result? The two species behaved in opposite ways. T. splendens started growing rapidly on alder leaves by efficient utilization of free amino acids and probably metabolism of other leaf tissue components, resulting in significant weight loss of leaves. T. marchalianum started growing on beech leaves, although slowly, by a more efficient absorption of free amino acids but with no effect on leaf decay. The data thus suggested a distinction to be made between the two species. Ex-

cess amounts of certain amino acids in the water promoted the growth of T. splendens on alder leaves, suggesting flexibility in habitat selection and presence of specific proteinase inhibitors on alder leaves. T. marchalianum was less compatible in the beech leaf environment and a more general metabolic inhibition seemed to prevent extensive growth of this species on beech leaves. There was at least one obvious similarity between the two species. Both demonstrated a key function for adjustment of protease activity to the concentration of free amino acids; T. marchalianum switched from high to low protease activity on alder leaves and T. splendens did the same on beech leaves in presence of excess amounts of free amino acids. This switching mechanism was described for whole leaf communities in a previous paper (Bengtsson subm.).

Excess amounts of free amino acids had another influence on nitrogen metabolism in fungi, especially on alder leaves. Both ammonia and, to a less extent, nitrate were taken up by the fungi from the water, but uptake was turned into a heavy exudation when concentration of free amino acids increased (Tab. 4). Inorganic nitrogen thus seemed to be an important nitrogen source for the fungi at low to moderate concentrations of free amino acids in the water but accumulated as waste products when the fungi metabolized excessive amounts of free amino acids. Thornton (1963) used T. splendens in one of his experiments with pure cultures of aquatic hyphomycetes and reported a 99 % utilization of NH_4 and NO_3 after 13 days incubation. Although it is hard to tell from this experiment whether exuded ammonia and nitrate was reabsorbed by fungi, it is notable that not even in a "nitrogen broth" did T. marchalianum develop any substantial mycelium on beech leaves, which is evidence for a strong growth inhibition.

Differences in some chemical key factors, such as nitrogen compounds, can thus not be eliminated as an explanation for the abundance of T. splendens on beech leaves and T. marchalianum on alder leaves. Both species have a nitrogen demand and use a highly flexible mechanism to provide for it. They use habitat specific proteases to hydrolyze leaf proteins but also absorb free amino acids and nitrate/ammonium from the water. The experiments clearly demonstrate a growth inhibition of T. marchalianum on beech leaves, and also bring up another phenomenon to be tested to explain the low abundance of T. splendens on alder leaves, namely interspecific competition.

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ENERGETIC COSTS OF AMINO ACID EXUDATION IN THE INTERACTION

BETWEEN THE PREDATOR GAMMARUS PULEX L. AND

THE PREY ASELLUS AQUATICUS L.

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ABSTRACT

The amphipod Gammarus pulex L. displayed aggregative behavior when stimulated by exudates from the isopod Asellus aquaticus L. in laboratory experiments, while the isopod avoided its predator by chemotaxis. Nonvolatile exudates from the amphipod significantly increased the respiration rate of the isopod. Exudation of free amino acids was greatly enhanced when both species were present in the same water and it was calculated that the increased exudation by the prey was equivalent to a loss of 4-5 % of its assimilated energy.

INTRODUCTION

Since MacArthur's formulation of the optimality theory (MacArthur and Pianka 1966) several pertinent contributions to behavioral ecology have been published (Krebs and Davies 1978). The theory has been developed within the framework of natural selection, which tends to produce individuals that maximize their inclusive fitness by optimal behavior. One of the main problems an animal is faced with is to obtain enough food without becoming food itself. Behavior developed for such interactions with other organisms requires energy and adds to total respiratory costs. Visual courtship displays and aggressive encounters that are important for maximal gene survival may also represent considerable respiratory expenditures (Stiles and Wolf 1970). The same holds for prey which are skillful at escaping, avoiding, and defending. Chemical defense developed by invertebrate and plant species is probably an evolutionally optimal strategy, although the short-term energetic costs of developing specific biochemical defense mechanisms may be large.

Defense behavior may involve release of other chemical compounds than those used to recognize and avoid a potential predator. Dissolved organic compounds that are exuded to some extent by most organisms may suddenly escape during the interaction and add to the energetic costs. In the case of a predator-prey relationship, loss of organic exudates probably represents a secondary effect of the organism's physiological mobilization pattern. Unless the exuded compound is a repellent which promotes the survival of the individual, exudation is a net metabolic cost which influences the equilibrium between costs and benefits of the behavior.

While a rather extensive literature deals with alarm and escape responses of marine invertebrates to predatory starfish odor and carnivorous snails (e.g. Atema and Burn 1975, Phillips

1977, 1978), few if any studies have been made on limnic invertebrates. I report here a sudden increase in amino acid exudation due to behavioral interactions between the prey Asellus aquaticus L. and the predator Gammarus pulex L., two species that frequently coexist in temperate running water systems. Normally, 80-90 % of the exuded amino nitrogen appears as ammonia and only minor amounts as amino acids (Dresel and Moyle 1950). As the prey recognizes the predator, however, a certain amount of amino acids is not metabolized but exuded from the body.

METHODS

Animals were caught in Höljeå, a small river in southern Sweden, brought to the laboratory and acclimated for one week at 15°C in circulating stream water. The animals were starved for 24 h in sterile, filtered stream water to remove gut contents and reduce amino acid excretion to a minimum. A three-way experimental design was used with 1000 ml bottles: 15 bottles contained 10 Asellus aquaticus, 15 bottles contained 10 Gammarus pulex and 15 bottles contained 5 Asellus and 5 Gammarus. The mixed species set was duplicated so that, in one of the experiments, the species were separated in the bottles by a plastic net. The experiments were run in existing light (in darkness Gammarus consumed Asellus) in sterile, filtered, 15°C stream water. Animals were allowed to acclimate in groups of five to the bottle environment for two hours before the experiments. The stream water was removed with animals left untouched and new stream water added to about half the bottle volume. The animals in two bottles were then randomly combined (e.g. 5A+5A, 5A+5G) to give the composition above. After 1, 2, 24, 72 and 215 hours, three bottles in each group were removed and the water was filtered (0.45 µm) and analyzed for free amino acids by gas chromatography (Bengtsson and Odham 1979).

To distinguish between visual and chemical stimuli for the exudation, another experiment was performed. Ten Asellus or Gammarus were added to jars containing 500 ml stream water and incubated at 15°C for 5 hours. The water was removed and purged for 10 min, and 50 ml was distributed to each of 10 jars. Five of the jars containing amphipod-stream water received five isopods each, and the remaining five jars received five amphipods each. The same additions were made to jars containing isopod-stream water. In addition, five control jars containing stream

water plus five animals were prepared for each species. All animals were previously acclimatized to stream water at 15°C. The jars were closed and incubated at 15°C for 5 hours. Oxygen concentrations were determined at the beginning and end of the experiment by a micro Winkler method.

A third group of experiments was conducted to compare the responses elicited by the prey when the predator was placed upstream and by the predator when the prey was placed upstream in a set of plastic tubes and troughs. In each experiment two troughs, 10x15x5 cm, were connected to each other by plastic tubing, 20 mm i.d. Drains were positioned centrally in the troughs which were supplied with running tap water (10-20°C) in PVC tubing (5 mm i.d.). A section of wider stoppered PVC tubing, 20 mm i.d. 60 mm length containing 20 animals of the species to be tested upstream, was inserted in the inflow to one of the troughs. In one experiment two sections of the wider PVC tubing were inserted in the same inflow, one with 20 *Asellus* preceding one with 20 *Gammarus*. Water flow to both troughs was 10 ml/min. 15 animals of the species to be tested downstream, previously starved for 48 h, were placed in each of the two troughs and observed for three nights. The number of animals in each trough was recorded at 6 h intervals.

RESULTS AND DISCUSSION

During the first two hours of incubation a rapid increase in free amino acid concentrations was observed (Fig.1). The increase was especially pronounced in water with both species present (independent of the plastic net), and the concentration peak was at least twice as high as the sum of the peaks from the single species. This suggests that visual or chemical contact between the prey and the predator gave rise to an enhanced amino acid exudation. The extent of amino acid exudation in the single species indicated the relative contribution of each species to exudation during the interspecific interaction. The contribution of *Asellus* was especially evident for the more complex amino acids - lysine, arginine, histidine and tryptophane (Tab.1). Interestingly, animals usually lack the enzymes responsible for synthesis of several of the amino acids that predominated in the exudation (leucine, lysine, histidine) (Meister 1972). Although this is an additional drawback of the exudation behavior when nutritional

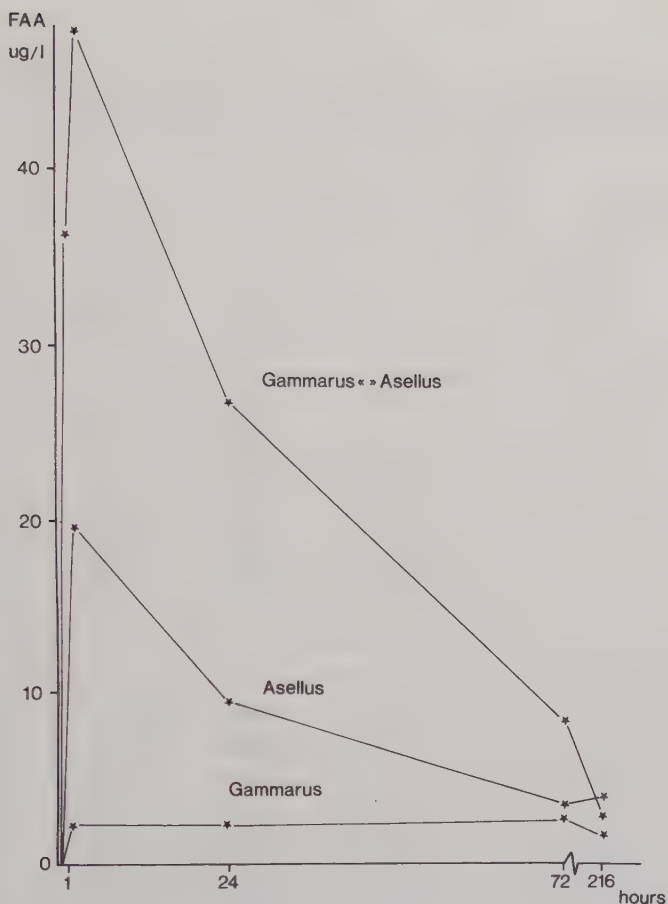


Fig.1. Exudation of free amino acids (FAA) from *Asellus aquaticus* L. and *Gammarus pulex* L. Each point represents the sum of the individual amino acid concentrations at one sampling time.

requirements are considered, the animals might compensate for this loss by utilizing hydrolyzed proteins synthesized by intestinal microorganisms.

This experiment does not explain the larger base level exudation from *Asellus* compared to *Gammarus*. It may depend on a combination of increased activity due to the handling of the material and differences in the exudation barrier of the exoskeleton. There are at least three possible reasons for the declining amino acid concentration following the rapid exudation in *Asellus* and

TABLE 1

Exudation of individual amino acids ($\text{ng} \cdot \text{l}^{-1}$) from *Asellus aquaticus* and *Gammarus pulex*. Mean ($n=3$) and standard deviation is given.

	1	2	24	72	9d
Gammarus	ala			83 $\pm$ 7	67 $\pm$ 4
	gly			26 $\pm$ 3	19 $\pm$ 7
	val				7 $\pm$ 2
	thr		228 $\pm$ 16	140 $\pm$ 15	97 $\pm$ 11
	ser	489 $\pm$ 22	414 $\pm$ 18	120 $\pm$ 8	134 $\pm$ 13
	leu	7 $\pm$ 3	35 $\pm$ 6	42 $\pm$ 4	19 $\pm$ 2
	ile				
	pro	232 $\pm$ 15	75 $\pm$ 8	240 $\pm$ 16	87 $\pm$ 7
	met		34 $\pm$ 2	47 $\pm$ 2	98 $\pm$ 4
	asp		232 $\pm$ 18	215 $\pm$ 12	149 $\pm$ 8
	phe	320 $\pm$ 19	341 $\pm$ 27	395 $\pm$ 18	315 $\pm$ 21
	glu	95 $\pm$ 11	54 $\pm$ 5	147 $\pm$ 11	164 $\pm$ 15
	tyr	60 $\pm$ 15	53 $\pm$ 4	24 $\pm$ 3	21 $\pm$ 1
	lys		84 $\pm$ 4	80 $\pm$ 6	8 $\pm$ 2
	arg	166 $\pm$ 25	340 $\pm$ 52	551 $\pm$ 65	160 $\pm$ 20
	his	499 $\pm$ 37	215 $\pm$ 26	168 $\pm$ 18	135 $\pm$ 21
	try	453 $\pm$ 32	146 $\pm$ 12	391 $\pm$ 28	217 $\pm$ 18
Asellus	ala				37 $\pm$ 2
	gly				
	val				
	thr				41 $\pm$ 5
	ser		124 $\pm$ 8	118 $\pm$ 7	135 $\pm$ 8
	leu		7 $\pm$ 2	10 $\pm$ 4	12 $\pm$ 4
	ile				
	pro	181 $\pm$ 4	237 $\pm$ 16	74 $\pm$ 2	115 $\pm$ 7
	met		62 $\pm$ 3	78 $\pm$ 5	143 $\pm$ 10
	asp				143 $\pm$ 18
	phe				
	glu	320 $\pm$ 20	72 $\pm$ 6	69 $\pm$ 6	57 $\pm$ 6
	tyr		156 $\pm$ 8	153 $\pm$ 10	142 $\pm$ 5
	lys	554 $\pm$ 105	1153 $\pm$ 63	35 $\pm$ 4	48 $\pm$ 6
	arg	8022 $\pm$ 543	3273 $\pm$ 412	1059 $\pm$ 123	2416 $\pm$ 338
	his	2035 $\pm$ 97	1787 $\pm$ 61	1114 $\pm$ 25	324 $\pm$ 11
	try	3379 $\pm$ 183	2516 $\pm$ 175	841 $\pm$ 64	359 $\pm$ 17
G + A	ala	280 $\pm$ 13	2625 $\pm$ 87	115 $\pm$ 6	141 $\pm$ 8
	gly	67 $\pm$ 5	521 $\pm$ 24	175 $\pm$ 15	34 $\pm$ 4
	val	252 $\pm$ 14	387 $\pm$ 18	88 $\pm$ 5	65 $\pm$ 4
	thr	80 $\pm$ 10	666 $\pm$ 42	103 $\pm$ 13	17 $\pm$ 8
	ser	173 $\pm$ 12	4548 $\pm$ 104	302 $\pm$ 18	453 $\pm$ 32
	leu	50 $\pm$ 8	9873 $\pm$ 403	517 $\pm$ 34	389 $\pm$ 34
	ile				
	pro	565 $\pm$ 21	705 $\pm$ 10	523 $\pm$ 23	248 $\pm$ 12
	met	168 $\pm$ 10	152 $\pm$ 12	183 $\pm$ 9	20 $\pm$ 3
	asp	54 $\pm$ 3	284 $\pm$	50 $\pm$ 5	70 $\pm$ 4
	phe	163 $\pm$ 8	174 $\pm$ 10	269 $\pm$ 15	595 $\pm$ 22
	glu	224 $\pm$ 18	341 $\pm$ 16	171 $\pm$ 19	105 $\pm$ 11
	tyr			62 $\pm$ 7	364 $\pm$ 19
	lys	17290 $\pm$ 300	7460 $\pm$ 146	9312 $\pm$ 412	1573 $\pm$ 86
	arg	8537 $\pm$ 612	9600 $\pm$ 817	8932 $\pm$ 789	3239 $\pm$ 140
	his	8100 $\pm$ 367	6740 $\pm$ 303	3401 $\pm$ 257	338 $\pm$ 18
	try	431 $\pm$ 28	3517 $\pm$ 105	2493 $\pm$ 84	792 $\pm$ 36

TABLE 2

Respiration of Asellus aquaticus and Gammarus pulex ($\text{mg O}_2 \cdot \text{l}^{-1} \cdot \text{d}^{-1}$) in modified stream water. Mean ($n=5$) and standard deviation is given.

	Respiration of <u>Asellus</u>	Respiration of <u>Gammarus</u>
in <u>Asellus</u> exudates	7.35±0.26	7.81±0.28
in <u>Gammarus</u> exudates	9.48±0.40	8.64±0.45
control (stream water)	7.22±0.21	7.76±0.17

Asellus+Gammarus: amino acids were consumed either by the animals or by microorganisms/protozoa attached to the exoskeleton (Nenninger 1948) or by both groups. The equilibrium between exudation and consumption of amino acids that was reached within few hours in the Gammarus population may also have been influenced by microorganisms.

Both Asellus and Gammarus increased respiration rate when exposed to amphipod exudates (Tab.2, $p < 0.01$ and $p < 0.05$, respectively, Kruskal-Wallis test). Since volatiles were removed by purging, less volatile compounds seemed to be responsible for the respiration change. The behavior of Asellus in Gammarus exudates was in agreement with the result on free amino acid exudation. The amphipods used in the experiment were of the same size but different sexes, which might explain the intraspecific dependence. The predator's lower contribution to amino acid exudation suggested in Fig.1 and Tab.1 was confirmed by the insignificant increase in respiration rate of Gammarus in Asellus exudates.

The predator was significantly attracted by water passing the prey (Tab.3). The prey displayed a weak response to water passing the predator, but the response was strong when a group of prey was placed upstream of the predator. This suggests that the exudation of chemical(s) by the predator, which stimulate the prey presumably to avoid capture, is largely initiated by the prey. What kind of chemical that emanate from the prey to stimulate the predator and from the predator to stimulate the prey is unknown but preliminary observations on the food preference in Gammarus pulex show that it is attracted by fungal mycelium rich in protein amino acids (Bengtsson, unpubl.). Though the heavy exudation of free amino acids in Asellus is a consequence of the response to a chemical stimuli from the predator, the base level exudation may be sufficient to attract the predator.

TABLE 3

Response of the predator *Gammarus pulex* and the prey *Asellus aquaticus* to each others exudates. Mean and SD of six observations. Some individuals stayed in the connecting tube.

Upstream test species	Downstream test species	Number of downstream test species preferring: tapwater	tapwater+exudates from upstream species	χ^2	P
<i>Asellus aquaticus</i>	<i>Gammarus pulex</i>	6.5 $\pm$ 1.1	21.5 $\pm$ 2.4	18.8	<0.002
<i>Gammarus pulex</i>	<i>Asellus aquaticus</i>	17.5 $\pm$ 4.2	11.0 $\pm$ 2.0	3.6	<0.5
<i>Gammarus pulex</i> ¹	<i>Asellus aquaticus</i>	26.5 $\pm$ 2.3	1.5 $\pm$ 1.8	54.5	<1.6 $\times 10^{-10}$

¹ 20 *Asellus* were placed upstream 20 *Gammarus*, which were placed upstream the test animals.

The attraction behavior to a food source in response to a chemical stimulus that was shown by Gammarus has been found with e.g. sea urchins (Strongylocentrotus droebachienses) (Garnick 1978) and with the mud snail Nassarius obsoletus in response to the bivalve Modiolus demissus and the gastropod Littorina littorea (Atema and Burd 1975). Simple organic compounds may be responsible for such stimuli. Woodbridge (1978) found that periwinkles, Littorina littorea, which usually aggregate on pieces of decaying sea weed, were attracted by certain sugars, mainly glucose.

Few attempts have been made to isolate and characterize compounds that induce aggregation or escape in aquatic environments. Pfeiffer and Lemke (1973) showed that the molecular weight of the alarm substance of minnows (Phoxinus laevis) was below 500, while a preliminary chemical analysis of the alarm substance of the snail Nassarius obsoletus indicated a molecular weight of about 100,000 or greater (Atema and Stenzler 1977). These examples and some few others reveal a tendency for molecular size distribution to be correlated to the specific habitat used. It may be hypothesized that these compounds belong to one of two categories. 1. Small, essentially hydrophilic compounds used by pelagic animals. These animals require compounds that are rapidly dissolved and transported in the water, regardless of how fast the molecules are degraded. 2. Long-chain molecules containing hydrophobic side-chains that prevent rapid degradation and help keep the molecule associated with the bottom water which is rich in organic matter with polar properties. Behavioral compounds used by bottom-living animals should thus be larger molecules.

The loss of organic molecules such as amino acids that contain high potential energy because of relatively small entropy represents an energetic cost to the individual. In order to roughly estimate the importance of amino acid exudation in the animal's energy budget, the standard heat of formation (ΔH_f°) (Cos and Pilcher 1970) for the different amino acids analyzed was used to calculate exudation energy. Assuming that only slight changes in volume and pressure occurred during the experiment, exudation energy may be compared with data on consumption and assimilation energy obtained with a bomb calorimeter (Tab.4). Provided that at least half of the exuded amino acids originate from Asellus (which is probably an underestimation), more than 1 % of the consumed energy (4-5 % of the assimilated) is lost during the course of interaction with the predator. The normal

TABLE 4

Comparison of consumption, assimilation and exudation energy in Asellus aquaticus and Gammarus pulex. (a) from Prus (1971), assuming uncrowded conditions and consumption evenly distributed over the day; (b) from Nilsson (1974), assuming animals larger than 10 mg; (c) calculated from ΔH_f° values in Cos and Pilcher (1970), based on exudation after 216 hours; (d) calculated from ΔH_f° values in Cos and Pilcher (1970), based on exudation after 2 hours.

	Consumption, 10^{-3}cal	Assimilation, 10^{-3}cal	Exudation, 10^{-3}cal	
			normal	interaction
Asellus aquaticus	51.3 ^a	13.1 ^a	0.3 ^c	0.6 ^d
Gammarus pulex	208 ^b	83.3 ^b	0.1 ^c	

loss of exudation energy for Asellus is at least 0.2 %, and for Gammarus 0.05 %.

Usually both Asellus aquaticus and Gammarus pulex exploit the same food resource, hyphomycetes (Bärlocher and Kendrick 1973, 1974, Kostalos and Seymour 1976, Rossi and Fano 1979), and at least in southern Sweden they have overlapping habitat niches. In laboratory experiments with different density levels and age groups, Asellus has been shown to be effectively preyed upon by Gammarus (Friberg, unpubl.), and it is argued that Asellus is more important for Gammarus as prey than as competitor. However, it may be difficult to separate the effects of prey defenses from those of predator preferences (Phillips 1977). Gammarus may prefer leaves with abundant mycelium over Asellus in laboratory choice experiments so that Asellus would be expected to be rare in its diet. Such food preference may be a result of the defensive responses of the mobile isopod.

Provided that Asellus can discover the presence of Gammarus at a short distance, the frequency of periods with enhanced exudation depends on the distribution and density of the populations. Although amino acids form an important part of the exuded material of all animals, not only due to their energetic value but also to their importance as biochemical precursors and essential nitrogen sources, other exudated compounds such as carbohydrates and organic acids probably contribute substantially to the exudation expenditures. Whether it is a general phenomenon that diffe-

rent types of interactions promote the outbreak of a "cold sweat" behavior by a potential prey is not known. As long as individual prey have a high probability to escape the predator by some chemical communication system, relatively high behavioral costs may be accepted.

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A MICROMETHOD FOR THE DETERMINATION OF METALS IN BIOLOGICAL

TISSUES BY FURNACE ATOMIC ABSORPTION SPECTROPHOTOMETRY

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ABSTRACT

A method was developed for determination of metals in samples of some few microorganisms weight. Samples were wet oxidized in small test tubes and an aliquot of the sample was automatically injected in a carbon rod atomizer. Precision and recovery was good for the analytical procedure. Application of the method to analysis of soil invertebrates showed a considerable variation in metal concentration between individuals from the same habitat.

INTRODUCTION

The recent development of furnace technology has resulted in an upswing for atomic absorption spectrometry in trace determination of environmental pollutants. By optimization of the instrumentation 1-10 pg can be detected for some metals in solution (Bertenshaw et al. 1981, Halliday et al. 1980) and this detection limit is sufficient for many environmental samples. Analysis in the lower ppb range usually requires preconcentration, which traditionally has been undertaken by solvent extraction and ion-exchange of tens and hundreds of milliliters (Wilson 1979). These techniques, however, have the disadvantage of increasing the time and complexity of the analysis. Little attention has been paid to problems associated with analyses of samples of very limited size, such as some few μg or μl (Koirtyohann et al. 1976, Sperling 1979). For analysis of very small sample sizes we have developed a microvolume technique which eliminates the disadvantages of preconcentration procedures. The purpose of the study was to provide a quick and reliable analytical method for metals in micro samples, with special emphasis on monitoring environmental pollution.

EXPERIMENTAL

APPARATUS

A Varian AA6 atomic absorption spectrophotometer was used with a Varian model CRA-63 carbon rod atomizer, modified with an electrode head model 90, a hydrogen lamp for background correction, a Varian ASD-53 autoinjector and a 255/MM Linear Instrument Corporation chart recorder. Peak height was determined with a peak reader PRM-6. Wet oxidation of samples took place in an electric heating block (Multi-block heater No. 2091). The aluminium block was drilled with seven mm holes. A Cahn model 25 automatic elect-

robalance (precision $\pm 0.1 \mu\text{g}$) was used to weigh the samples.

REAGENTS AND STANDARDS

Analytical grade nitric acid was used for digestion of samples. Stock solutions of metals (10,000 ppm) were prepared separately with Merck Titrisol standard. Stock solutions were diluted with high purity 0.1 M hydrochloric acid, to make the solutions contain 10.0 ppm of the metals. Calibration standards were prepared by serial dilution with 0.1 M hydrochloric acid immediately prior to determination. The precision of the instrument and the calibration was recorded for calibrated standards ($n=3$) in the range 0-160 ppb of Pb and Cu.

PROCEDURE

Prewashed samples were dried overnight on aluminium cups or small beaker covers in a desiccator. The samples were weighed and transferred to small test tubes (Pyrex, 3.7 mm i.d., 9.0 mm length, 2.3 mm thickness). The test tubes were washed in 5 % Deconex, rinsed in distilled, deionized water, soaked in 1 M hydrochloric acid overnight and finally rinsed in distilled, deionized water. Fifteen μl nitric acid was added and the samples were heated in the aluminium block at 95°C until dry. A procedural blank with 15 μl nitric acid was run each time to compensate for reagents and test tube contaminations. If dark residues remained in the tubes, another 15 μl nitric acid was added and consequently a blank with 30 μl nitric acid was run. The samples were stored in a refrigerator until analysis.

At least one hour ahead of analysis the residue was dissolved in 50.0 μl 0.1 M HCl and mixed by inserting 1 ml of the air through a plastic tubing into the solution. The test tubes were placed in microcups in the sample carousel and a 5 μl sample was injected automatically. For lead, the carbon rod operating parameters were $\approx 85\text{--}95^{\circ}\text{C}$ drying for 40 s, $\approx 450^{\circ}\text{C}$ (5 V) ashing for 20 s and $\approx 2400^{\circ}\text{C}$ (8 V) atomizing for 2.5 s. Peak heights were used for quantification. Care was taken during the procedure to avoid airborne contamination.

The accuracy of the procedure was determined from 50 μl of calibration standard (0-160 ppb) added to the test tubes and eva-

porated in a desiccator at reduced pressure. The residue was taken through the procedure and the peak height for Pb and Cu compared with the peak height for the elements in pure standards. The precision and effectiveness (in terms of recovery) of the analytical method was determined by adding 10 μ l of a Pb or Cu solution to a sample of the waterflea, Daphnia magna, corresponding to 25 μ g dry substance, in a test tube, followed by drying of the sample in an oven at 60°C

RESULTS AND DISCUSSION

The peak height for calibration standards was linearly related to the concentration within the range 0-160 ppb ($r=0.999$). Except for the lowest concentration used, 10 ppb, the procedure showed a good accuracy, as exemplified with Pb (Tab.1). There is a slight underestimate of the true value, due to losses of metal in the procedure, i.e. because of absorption to the glass, especially in the lower range, but in most cases the true value is within the 95 % confidence interval of the sample mean. Recovery of spiked Cu and Pb from samples of Daphnia magna was better than 97 % (Tab.2). The recovery was lower when small amounts of metals were used for spiking.

TABLE 1

Accuracy of analytical procedure for determination of Pb in calibration standard. SD = standard deviation, CV = coefficient of variation, CI = confidence interval.

Expected concentration,ppb	Measured concentration,ppb	SD	CV	95 % CI
10	8.5	0.5	0.06	7.3-9.7
20	18.0	1.0	0.06	15.5-20.5
40	38.0	2.0	0.06	33.0-43.0
80	78.5	1.5	0.02	74.8-82.2
160	158	2.0	0.01	153.0-163.0

TABLE 2

Mean recovery (n=5) of Cu and Pb from spiked samples of a water flea, Daphnia magna.

Added amount, ng	Recovery, %	
	Cu	Pb
0.5	97.6	97.3
1.0	98.1	97.5
2.0	98.4	98.0
4.0	99.6	98.2
8.0	100.0	99.5

The limit of detection was derived from the standard deviation of the procedural blank. It is given by the equation

$$d = b + ks_b$$

where d = detection limit, b = blank concentration, s_b = standard deviation of the blank and k = multiplier corresponding to the required degree of confidence. As concentration measured = concentration of blank + concentration of digested material (x), it follows, that for a quantity to be detectable,

$$x \geq ks_b$$

The mean value of Pb and Cu in the procedural blank was 3 ppb with a standard deviation of 0.7 ppb. The multiplier in a t-distribution with 90-95 % confidence limits is approximately 3, which gives a practical detection limit of $3 \times 0.7 = 2.1$ ppb.

Due to the very small sample size, interferences from matrix material was virtually eliminated and samples were closely similar in composition to prepared standards. Atomization of lead, which is severely suppressed by inorganic matrix constituents (Bertenshaw et al. 1981, Regan and Warren 1978, Thompson et al. 1977) was made at a high temperature. The high temperature accentuated one of the weaknesses of the graphite tube, namely its short lifetime (usually less than 75 injections), although the lifetime can be increased by adding lanthanum chloride to the sample (Bertenshaw et al. 1981, Thompson et al. 1977).

The usefulness of the carbon rod atomizer for routine analysis was further limited by irreproducibility of injections into a new tube. The coefficient of variation was typically larger than 1.0 for the first 10-15 injections, but when properly conditioned, the carbon rod gave good precision.

Approximately 50 microsamples can be processed in our laboratory each week by a full time, well trained technician. Sample drying takes 1-2 days, depending on the size of the tissue, one day is required for the oxidation, and about 20 samples can be determined for one element per day, provided triplicate injections are made for each sample. The precision for triplicate injections is demonstrated by the determination of Pb in a single specimen of the springtail (Collembola) Onychiurus armatus, from an uncontaminated laboratory culture and from a metal contaminated field site (Fig.1). The specimens had a length of 1.13 mm and 1.07 mm, and a weight of 24.7 μg and 21.8 μg , respectively. Four to five times higher concentration was found in the specimens from the contaminated site.

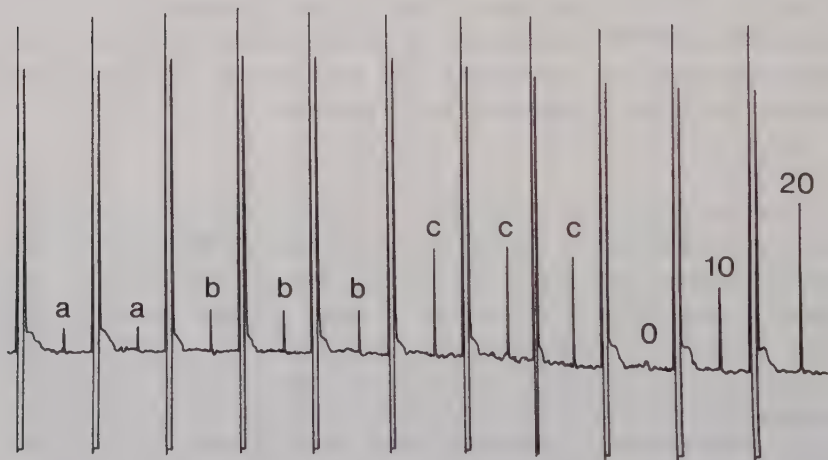


Fig.1. Microdetermination of lead; a = procedural blank, b = specimen of Onychiurus armatus from an uncontaminated laboratory culture, c = specimen of O. armatus from a metal contaminated field site. 0, 10 and 20 (ppb) are peaks for calibration standards. The concentration in (b) specimens was calculated to 5.88 $\mu\text{g/g}$ and for (c) specimens to 25.89 $\mu\text{g/g}$.

TABLE 3

Mean (ng/mg), standard deviation (SD) and coefficient of variation (CV) for Pb in muscle tissue from the earthworm Lumbricus terrestris, grown in natural soil amended with different amounts of Pb, and for Pb and Cu in the springtail Onychiurus armatus feeding on an uncontaminated and metal contaminated fungus in laboratory culture.

<u>L.terrestris</u>			<u>O.armatus</u> Uncontaminated fungus			<u>O.armatus</u> Contaminated fungus		
Mean	SD	CV	Mean	SD	CV	Mean	SD	CV
Pb			Pb			Pb		
5.6	3.3	0.58	6.5	5.0	0.77	512.0	21.0	0.04
8.7	3.6	0.41						
29.9	14.9	0.50	Cu			Cu		
47.1	15.9	0.34						
80.0	29.9	0.37	75.0	35.0	0.47	652.0	240.0	0.37

A large number of tissue and whole body samples of small invertebrates have been analyzed for Pb, Cd, Cu, Ni, and Zn in our laboratory. A considerable natural variation was found in the samples, which is illustrated in Tab.3. While the coefficient of variation normally is less than 6 % for the analytical method (Tab.1), coefficients of variation close to or above 50 % were not uncommon for invertebrate tissues. Some errors in the determinations were obvious, such as the presence of microscopic soil particles on the surface of washed animals, but the variations in metal concentration in specimen from laboratory cultures, e.g. O.armatus in Tab.3, are difficult to explain unless assuming differences in feeding behaviour, metabolic activity, etc. between animals. This concept needs serious attention in the design of sampling program for laboratory experiments and environmental monitoring projects.

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GROWTH CHANGES CAUSED BY METAL UPTAKE IN A POPULATION OF

ONYCHIURUS ARMATUS (TULLB.) (COLLEMBOLA) FEEDING ON

METAL POLLUTED FUNGI

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ABSTRACT

The effects of uptake of copper and lead on the growth of *Onychiurus armatus* (Collembola) were studied in laboratory experiments. Specimens were reared in petri-dishes, supplied with a soil borne fungus *Verticillium bulbillosum*, which was grown on a substrate contaminated with different amounts of metals. Growth of specimens of *O. armatus* was measured in the F1- and F2-generations. Growth data were fitted to a growth model and the best estimates of growth rate and average maximum length were calculated and compared for different metal contaminations.

The fungus accumulated metals efficiently, reaching levels of 1,000 to 3,500 ppm on substrates containing 15 to 300 ppm metals. *O. armatus* concentrated metals during the first two weeks of its life cycle, and then excreted metals towards a steady state level. Copper concentration in the body was higher than lead concentration, and the metals behaved antagonistically when mixed. Neither metal was accumulated by Collembola, and equilibrium concentrations of copper varied from 150 to 600 ppm.

Growth rate and average maximum lengths were higher at a moderate metal pollution, both in F1 and F2, and significantly reduced on substrate containing 90 ppm metal or more. The lowest concentration of copper and lead in specimens with significantly reduced growth rate was about 200 ppm. This concentration was also found in a field population in the vicinity of a brass mill. The consequences of reduced growth rate on survival and reproduction in a population are mentioned.

INTRODUCTION

With the increasing concern about environmental pollution some work has been carried out on the influence of modern technology on the abundance of soil animals. Emissions from smelters and chlor-alkali works tend to decrease the abundance of most soil animal species and also to increase the body burden of metals in animals (Bull et al. 1977, Strojjan 1978). Accumulation and/or enrichment of metals have been documented in several soil animal groups both in field and laboratory studies (Ireland and Richards 1977, Czarnowska and Jopkiewicz 1978, Helmke et al. 1979, Joosse and Buker 1979, Williamson 1979, Hartenstein et al. 1980 and references in them), but some invertebrates evidently can survive high concentrations of certain metals in the body (Williamson and Evans 1973, Beeby 1978). Although high concentrations of metals seem to be necessary for significant reduction of population densities, lower concentrations may affect growth and population characteristics such as migration and reproduction that may have long-term influences on the density.

A deeper knowledge of population dynamics of soil animals and of the interactions between soil animals and microorganisms, is important for interpretation of the long-term effects of metal pollution on decomposition processes. This would, as suggested

by Tyler (1972), increase the accuracy of the predictions of long-term consequences on primary production due to limitation of the cycling of plant nutrients.

The purpose of this work was to study the uptake of lead and copper by the collembolan, Onychiurus armatus (Tullb), from its contaminated food, the hyphomycete Verticillium bulbillosum (W. Gams and Malla), and the impact of the metals on the growth of the animals.

MATERIAL AND METHODS

REARING CHAMBERS

A rearing chamber was made by placing two plastic cups ($\varnothing$ 15 mm, depth 6 mm) in a petri-dish. The petri dish was filled up to the top of the cups with a mixture of plaster of Paris and pulverized, activated carbon (9:1 w/w) (Goto 1960). The solidifying plaster surface was carefully smoothed to prevent the appearance of fissures, where the Onychiurus would hide their eggs. When the plaster had solidified, the plastic cups were removed, leaving holes that would fit the feeding cups (see "Feeding cups"). The surface of the plaster was cleaned with a brush and a stream of distilled water. The rearing chambers were sterilized at 80°C during four days, the first two days with the surface of plaster covered by 95 % ethanol.

FEEDING CUPS

After sterilization during at least two days in 95 % ethanol 20 plastic cups prepared for ten rearing chambers, were placed in a petri-dish and irradiated with UV-light in a sterile cabinet for 24 hrs (12 hrs on each side of the cups).

Solutions of $\text{Pb}(\text{NO}_3)_2$ and $\text{Cu}(\text{NO}_3)_2$ (30, 90, 300 ppm of each, and 0+0, 30+30, 90+90, 300+300 ppm Pb and Cu combined) and an agar solution (6 % Bacto-agar, 6 % Oxoid malt extract, 100 ppm $\text{KNO}_3\text{-N}$ and 100 ppm asparagine-N) were autoclaved separately for 15 min at 110°C; a mixture of metal ions and agar would have chelated at temperatures above 100°C.

When the solutions had cooled below 60°C each metal solution was mixed with an equivalent volume of agar and 30 ppm chlor-tetracyclin was added, reducing the metal concentrations in the

metal-agar solution to half of those mentioned above. Each broth was then poured into the petri-dishes with the plastic cups. A soil borne fungus was inoculated aseptically. After one week's incubation the fungus covered the surface of the metal-agar and the feeding cups could be removed and transferred to the rearing chambers.

FOOD PREFERENCE

The food preference of *O. armatus* was determined by testing seven species of fungi, viz. Mortierella isabellina (Oudem.), M. ramanniana (Moeller), Mucor silvaticus (Hagem), Mycelium radicis atrovirens (Melin), Penicillium spinulosum (Thom), Trichoderma viride (Pers.) and Verticillium bulbillosum (W. Gams and Malla). They are all common in mor horizons in spruce forests. Each species was inoculated in feeding cups (0+0 ppm Pb + Cu) and tested in different rearing chambers, each with five adult *O. armatus*. The collembolans had been extracted from litter - mor from a spruce forest, 20 km W of Gusum, SE Sweden, using a modified Macfadyen extractor. The rearing chambers were moistured with distilled water and then wrapped in plastic bags to maintain near 100 % RH. Due to water condensation the lid of a rearing chamber had to be exchanged when the eggs were counted. To prevent contamination a burner producing a vertical air stream was placed close to the microscope.

The estimated grazed area of the fungi and the number of eggs produced were used to determine the food preference.

Grazing and egg production were greatest when the collembolans were maintained on V. bulbillosum and M. ramanniana. The former species was used for growth and metal uptake experiments as M. ramanniana failed to grow on agar containing 150 ppm Cu, 45+45 or 150+150 ppm Pb + Cu. The very high metal concentrations did not seem to seriously affect V. bulbillosum, but a slight decrease of growth rate (one day's delay to reach the edge of the feeding cup) was found at 150 ppm Cu and 150+150 ppm Pb + Cu. This high degree of tolerance to metals is probably connected with different mechanisms to protect physiological processes, such as metal complexation and precipitation at the cell surface (Talbert and Johnson 1967, Antonovics et al. 1971).

Food preference experiments may be misleading, but since the fungi tested and the two species preferred by *O. armatus* were fre-

quently found in the same soils as this collembolan species (Nordgren pers.comm.) and since fungi also have a high content of important nutrients such as amino acids (Bent and Morton 1964) the use of V.bulbillosum as experimental food and metal source seems to be justified.

HATCHING PROCEDURE

600 adult Onychiurus spp. from the spruce forest mentioned above were transferred to 20 rearing chambers equipped with feeding cups containing V.bulbillosum growing on agar (0+0 ppm Cu + Pb). These individuals constituted the P-generation. Twenty randomly chosen Onychiurus were determined to species. All were O.armatus (Tullb.).

The number of eggs was counted daily in a microscope (10-80X). All eggs (none more than one day old) were transferred, in groups of 30, to 60 rearing chambers, six for each metal concentration. When the eggs were moved from one chamber to another, a bent, flattened inoculation needle was used. The needle was sterilized (flamed, cooled in ethanol and flamed again). A drop of distilled water was attached to the needle and the eggs were easily adsorbed.

The chambers were kept in darkness at 20°C and moistured with distilled water once a week. During egg-counting the chambers were exposed to daylight for about ten minutes. The feeding cups were renewed once a month.

The day when half of the fertile eggs were hatched was defined as the hatching date of the batch. The newly hatched O.armatus constituted the F1-generation.

To study the effects of metal on the F2-generation, one of the metal series was chosen. Eggs from about 110 days old F1-individuals reared in chambers of the Pb-series were transferred to new rearing chambers (six for each concentration). Eggs produced at 0 ppm Pb were transferred to 0 ppm Pb chambers, 15 ppm Pb to 15 ppm Pb chambers etc. When hatched they constituted the F2-generation.

The average egg production of the P-generation was low (0.05 eggs.animal⁻¹.week⁻¹) and ceased after one month of incubation at 20°C. Changing the incubation temperature or the population density did not stimulate the egg production. Not until the temperature was subsequently decreased to 4-6°C for 1-2 days and in-

creased to 20-22°C for 1-2 days, the egg production started and after two weeks 2.0 eggs·animal⁻¹·week⁻¹ were produced. The egg production decreased after another two weeks to a constant level, 0.5 eggs·animal⁻¹·week⁻¹. The average hatching was high, 78 % (range 34 - 100 %) and in general hatching took place after 14-15 days.

GROWTH

The lengths of the specimens of the F1- and F2-generations were measured in a microscope 0-2 days after hatching by an ocular micrometer at 30X magnification. Additional measurements were made once a week during the first seven weeks and in weeks 9, 11, 13, and 17. The lengths of ten randomly chosen individuals from each rearing chamber were determined at each time. The length of an individual (expressed in units; 1 unit = 0.0323 mm) was defined as the distance from the first segment of thorax to the end of the anal spines.

A computer program was written to fit the data to the equation:

$$L_t = L_{\infty}(1 - e^{-k(t-t_0)})$$

where

L_t = length of the individual at time t

L_{∞} = the asymptotic length

k = growth rate

t_0 = the hypothetical negative time estimated from hatching date for an individual of the length 0

This equation was first suggested by von Bertalanffy (1960) to describe the increase of lengths in populations of vertebrates and Crustacea.

The derivatives of the equation were specified in the program to facilitate the calculations of L_{∞} , k and t_0 with the least square method on the regression using a Gauss-Newton algorithm (Jennrich and Sampson 1968).

To test for statistical differences between the growth curves, the specific growth rate was plotted on reciprocal length. Specific growth rate was defined as the rate of growth divided by the length and estimated as $\ln L_2 - \ln L_1 / \Delta t$, where L_1 = length at the beginning of the time interval, Δt . The regression for each test

population was tested for linearity and the regression lines compared by analysis of covariance.

LENGTH - WEIGHT RELATION

About 150 newly extracted O.armatus were separated into six size classes on length basis. They were killed by deep-freezing and their lengths were determined individually. The individuals were dried for two days in a desiccator. Each size class was weighed on a Mettler micro-balance ($\pm 5 \mu\text{g}$) and the mean weight per individual and size class was calculated.

The length from the first segment of thorax to the anal spines was proportional to the square root of the weight ($r = 0.999$). The relative increase in length was slightly overestimated since the length of the head, which changes little during the growth, was not included in the measurement. A more accurate estimate of the weight of O.armatus was achieved by length measurement of single individuals than using a micro-balance.

METAL CONTENT OF FUNGUS

About 0.2 mg hyphae (dead and alive) was taken from two weeks old colonies of V.bulbillosum growing on metal-agar of different concentrations (see "Feeding cups"). The mycelium was transferred to aluminium cups ($\varnothing$ 14 mm, depth 2 mm, wt ca. 0.07 g), dried for two days in a desiccator and weighed on the micro-balance. The mycelium was transferred to glass microtubes ($\varnothing$ 3.8 mm, length 20 mm), dissolved in 50 μl conc. nitric acid and digested by heating. The acid was evaporated and the tubes were stored in a refrigerator at 4°C until the day of metal analysis (see "Metal content of Collembola"). Two duplicates were analysed for each metal concentration.

METAL CONTENT OF COLLEMBOLA

From a separate series of 40 rearing chambers (five for each metal concentration) O.armatus were taken at the age of 0, 1, 2, 4 and 8 weeks, five individuals each time from each chamber. The individuals were killed and the length of each individual was determined. Those from the same chamber were transferred to a microtube, digested and stored as above.

Immediately prior to metal analysis, 200 μ l 0.10 M hydrochloric acid was added to the microtube and the content was mixed by bubbling one ml air through a thin plastic tubing. The samples were analysed using a Varian AA6 atomic absorption spectrophotometer, equipped with a Varian CRA-63 carbon rod atomizer, modified with an electrode head model 90. Spectral lines used were 217.0 nm (Pb) and 324.7 nm (Cu), ashing temperature 450°C (5.5 V) for 20 sec and atomizing temperature 2,100°C (8 V) for 2 sec. The microtubes were placed in special sample cups in a Varian autoinjector (ASD-53). Standard solutions (160+160, 80+80, 40+40, 20+20, 10+10, and 0+0 ppb Pb + Cu) were made in 0.10 M hydrochloric acid from metal nitrate solutions containing 10,000 ppm Pb or Cu, respectively.

RESULTS

METAL UPTAKE IN THE FUNGUS

The concentration of metals was considerably higher in V.bulbillosum than in the contaminated nutrient broth (Tab.1). If a correction was made for the high water content of the nutrient broth (96 %), accumulation, defined as the ratio of metal concentration (on dry wt basis) in mycelium to metal concentration in the substrate, varied between a factor 8 and 0.6. The accumulation was almost inversely proportional to the concentration in the agar (Tab.1).

As the concentration of a single metal in the nutrient broth was increased, successively higher concentrations were found in the mycelium, reaching a maximum of about 3,000 ppm. A high concentration of a combination of metals in the broth seemed to result in a comparatively lower content in the mycelium. The transport mechanisms for metals in the mycelium seemed to have different affinities for different metals, single or in combination, since copper was retained to a greater extent as a single contaminant than in combination, while the opposite was true for lead.

The reduced metal content of the mycelium at 150+150 Pb + Cu (Tab.1), which was repeated with several cultures, is noteworthy and difficult to explain unless assuming antagonism between the metals.

TABLE 1

Concentrations of lead and copper in the mycelium of *Verticillium bulbillosum* grown on metal contaminated substrate and accumulation of the metals by *Verticillium bulbillosum*. Mean (n=2) and standard deviation are given.

Metals added to substrate (ppm)		Concentration in mycelium (ppm)		Conc. in <i>V.bulbillosum</i>
		Pb	Cu	Conc. in agar (d.w.)
0	Pb+Cu	8 $\pm$ 8	95 $\pm$ 76	
15	Pb	1068 $\pm$ 228		4
45	Pb	1096 $\pm$ 215		1.5
150	Pb	3089 $\pm$ 1		1.5
15	Cu		1296 $\pm$ 128	5
45	Cu		2608 $\pm$ 125	3
150	Cu		3245 $\pm$ 580	1.5
15+15	Pb+Cu	2404 $\pm$ 149	1512 $\pm$ 170	8
45+45	Pb+Cu	3410 $\pm$ 590	752 $\pm$ 54	3
150+150	Pb+Cu	1937 $\pm$ 578	1017 $\pm$ 580	0.6

METAL UPTAKE IN ONYCHIURUS

The concentration of metals in *O.armatus* changed in a distinct pattern, characterized by a short uptake phase, when most of the available binding groups such as sulphhydryl and carboxyl groups, were occupied, and a longer release phase, when detoxification processes released metals to a certain steady state level (Fig.1). The uptake phase was generally extended when the food was more and more contaminated and was slightly shorter when both metals were present in the fungi than singly. The release phase was slower for copper than for lead and the "equilibrium" level consistently higher. The equilibrium level increased when the concentration of metals increased in the mycelium but was more closely related to the metal concentration in the nutrient broth than in the mycelium.

The concentration of single metals in *Collembola* was generally lower when both metals were present in the food (Fig.1). None of the metals was accumulated since the ratio of metal in *O.armatus* to mycelium was less than 1.0 (Tab.2). However, copper was retained by *O.armatus* to a larger extent than lead when both metals were added to the substrate. The fungi and *Onychiurus* thus seemed to discriminate between the two metals in opposite ways (cf. Tab.1).

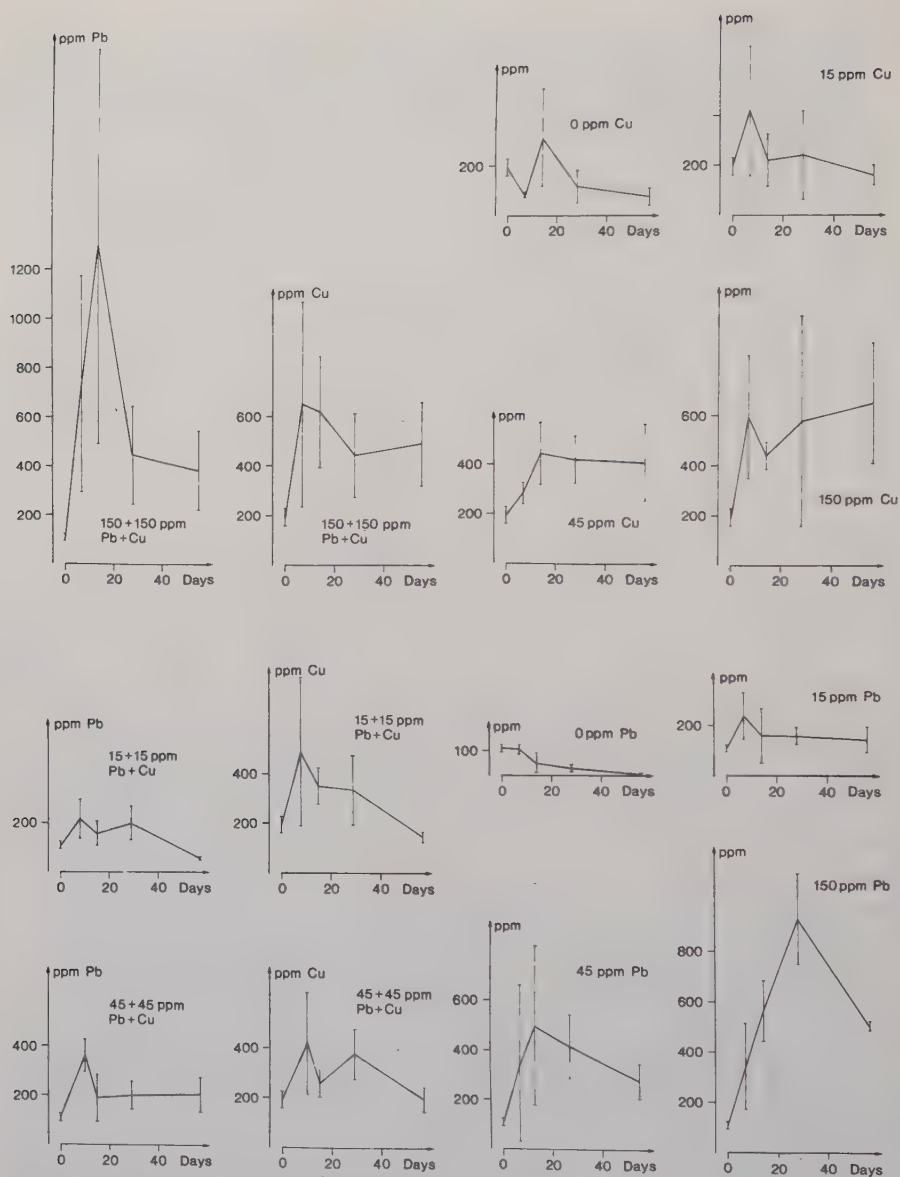


Fig.1. Concentration of copper and lead in *O. armatus* at different metal concentrations in the substrate of their food, *V. bulbiliosum*. Mean ($n=5$) and standard deviation are shown.

TABLE 2

Ratio between concentrations of metals in O.armatus feeding 55-57 days on V.bulbillosum growing on metal-agar solutions, and in V.bulbillosum.

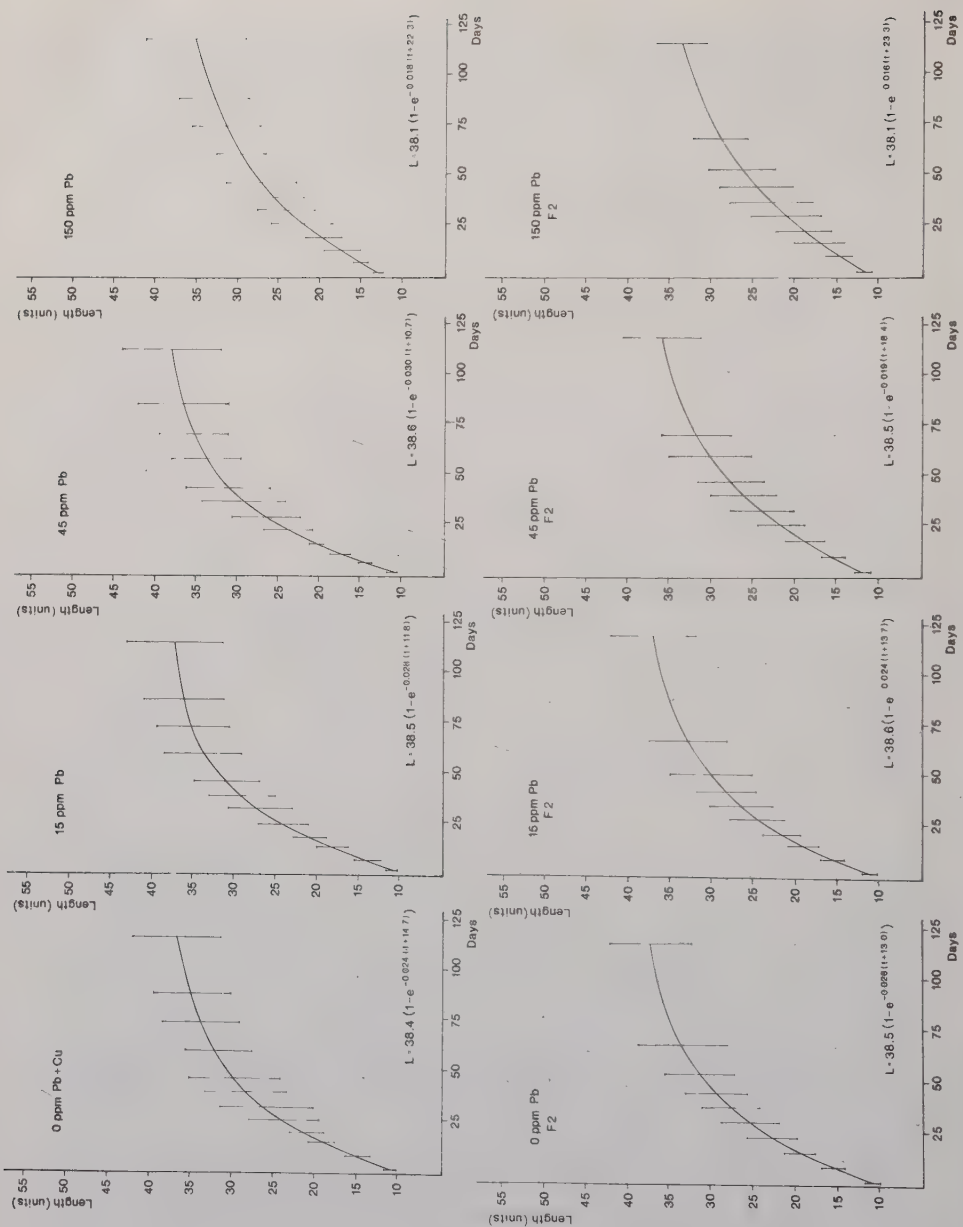
Metal concentration in substrate (ppm)	Concentration in <u>O.armatus</u> Concentration in <u>V.bulbillosum</u>	
	Pb	Cu
15 Pb	0.14	
45 Pb	0.25	
150 Pb	0.17	
15 Cu		0.13
45 Cu		0.16
150 Cu		0.20
15+15 Pb+Cu	0.02	0.10
45+45 Pb+Cu	0.05	0.26
150+150 Pb+Cu	0.20	0.50

The means for metal concentration in Onychiurus were significantly increased for increased metal concentrations in the agar, regardless of rearing time ($p < 0.005$, $F = 40.75$ for lead, 15.48 for copper, 20.37 for lead in Pb + Cu, and 9.51 for copper in Pb + Cu).

GROWTH OF O.ARMATUS

The non-linear model for growth of length versus age was fitted to the data (RMS less than 20, Tab.3) and the estimated parameters for growth rate and theoretical mean maximum length could be used for comparison of metal effects. Growth rate (k) was higher at a certain enhanced total metal concentration in the broth (30 and 45 ppm metal) and decreased at high concentrations (150 and 300 ppm metal) (Fig.2). O.armatus grown on slightly contaminated food thus reached maximal length faster than either in the control or on the most contaminated food.

The same pattern of growth differences was achieved using the second estimated parameter, theoretical mean maximum length, L_{∞} (Tab.3). Largest lengths were calculated for the populations grown on 30 and 45 ppm metal and the shortest ones were found for populations on 150 and 300 ppm metal. While lead and copper affected the growth rate in a very similar way, copper alone made the L_{∞} considerably shorter. The F2-populations reached the same



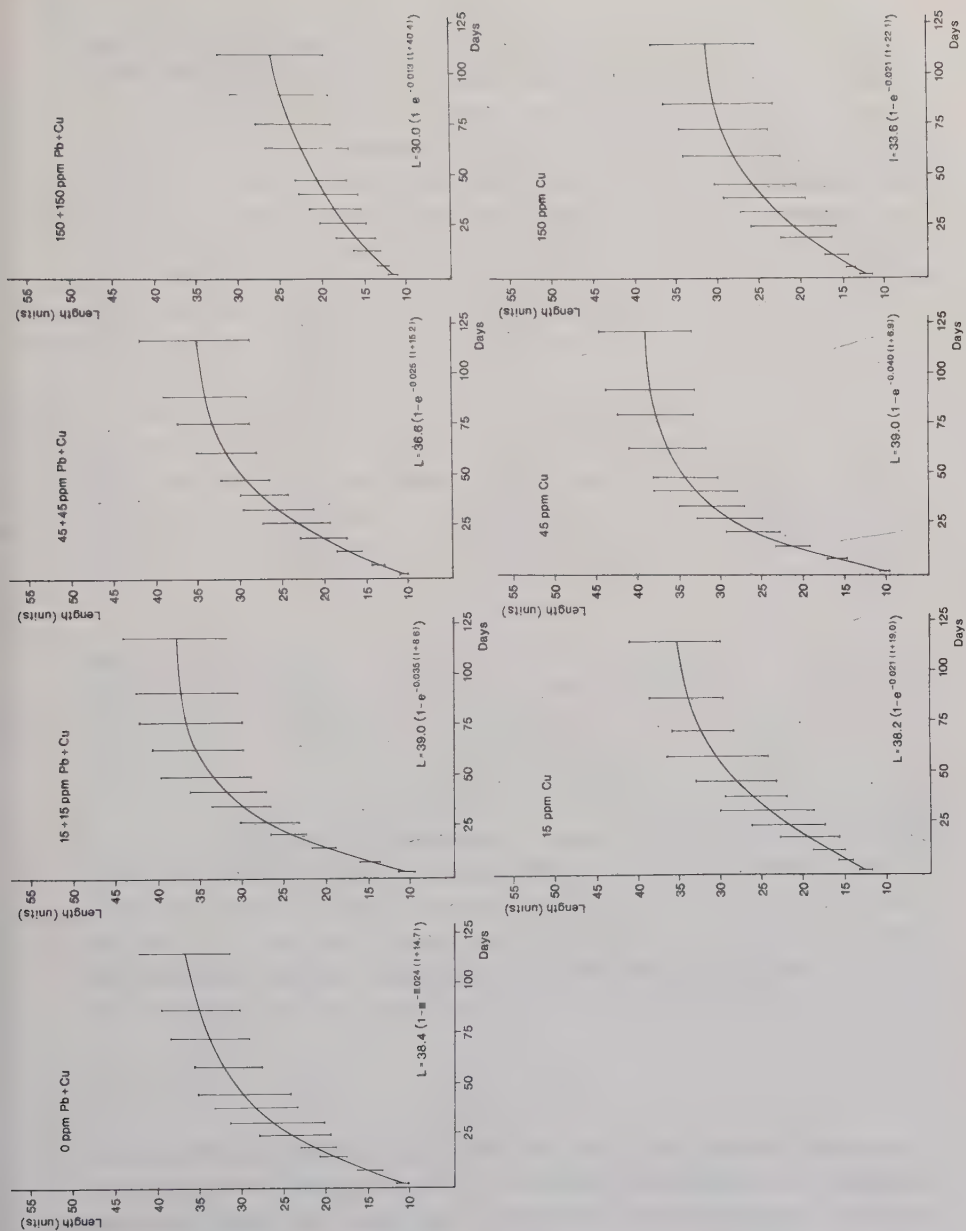


Fig.2. Growth of *O. armatus* fed upon *V. bulbiliosum* growing in substrates of different metal concentrations. Data were fitted to the growth equation: $L_t = L_{\infty}(1 - e^{-k(t-t_0)})$ and the best estimate of the asymptotic length, L_{∞} , the growth rate, k , and t_0 are given. Mean length and standard deviation were plotted for the best estimate. (1 unit = 0.0383 mm).

TABLE 3

Residual mean squares (RMS), theoretical mean maximum length L_{∞} , and growth rate, k , for fitting length of O.armatus to the theoretical growth model.

Concentration in substrate (ppm)		RMS	L_{∞}	k
F_1 -generation				
0	Pb+Cu	16.94	38.4	0.024
15	Pb	14.86	38.5	0.028
45	Pb	14.08	38.6	0.030
150	Pb	12.88	38.1	0.018
15	Cu	15.39	38.2	0.021
45	Cu	17.95	39.0	0.040
150	Cu	19.74	33.6	0.021
15+15	Pb+Cu	19.20	39.0	0.035
45+45	Pb+Cu	11.72	36.6	0.025
150+150	Pb+Cu	8.31	30.0	0.013
F_2 -generation				
0	Pb	11.91	38.5	0.026
15	Pb	11.80	38.6	0.024
45	Pb	10.99	38.5	0.019
150	Pb	11.12	38.1	0.016

average maximum length as the F_1 -populations, but the growth rates were lower in the metal contaminated substrates.

The specific growth rate of O.armatus was significantly reduced in 150 and 300 ppm of metals while lower concentrations of lead in F_1 made no significant difference (Tab.4). All combinations of metals changed the specific growth rate significantly, and in F_2 the limit for significant reduction was at 45 ppm Pb.

DISCUSSION

Collembola are not entirely detritus-feeders (Singh 1969, B  d-varsson 1970, Anderson and Healey 1972, McMillan 1975) but also consume microorganisms. Conflicting evidence has been published on the relative importance of microorganisms in collembolan food, and at least some differences are due to different extraction techniques of the animals from the soil (Joosse and Testerink 1977). It seems likely that different species channel their feeding efforts into different kinds of food, e.g. detritus, bacte-

ria, algae, and different species of fungi depending on habitat preference and competitive ability.

Some experimental results justify the choice of fungal species for this experiment. Snider (1974) reported a hatching success of 67.7 % at 21°C for O.armatus maintained on brewer's yeast. The high hatching percent in our experiment (a mean of 78 %) is probably due to the nutrient quality of the fungi. The relation between length and weight also speaks in favour of using V.bulbilosum as food. The weight of newly hatched individuals (total length 0.52 mm, 1.16 µg) corresponds closely to data reported by Petersen (1975), 1.06 µg. The average weight for large animals in our experiment (total length 1.87 mm, 48.67 µg) was higher than that found by Petersen (1975) for specimens extracted from field soils (31.86 µg), but lower than for those reared in laboratory (61.48 µg). It seems reasonable to assume that the unpredictable distribution of high quality food in a field soil accounts for the lower average weight in the field population.

TABLE 4

Analysis of covariance for the effect of metal contamination on the specific growth rate of O.armatus. The regression of the specific growth rate on the reciprocal length for populations reared on a substrate with no metals added was compared with regressions for populations reared on metal contaminated agar.

Metal concentration in agar (ppm)		Comparison of slopes	
		F	p
F ₁ -generation			
15	Pb	0.06	ns
45	Pb	0.12	ns
150	Pb	4.34	<0.05
15	Cu	3.99	<0.05
45	Cu	1.38	ns
150	Cu	15.64	<0.01
15+15	Pb+Cu	3.22	<0.1
45+45	Pb+Cu	4.98	<0.05
150+150	Pb+Cu	96.9	<0.01
F ₂ -generation			
15	Pb	0.52	ns
45	Pb	5.76	<0.05
150	Pb	12.43	<0.01

The calculated growth curves for O.armatus correspond to the theoretical model suggested by von Bertalanffy (1960) and are similar to those characteristic of O.furcifer (Börner) (Petersen 1971). Thibaud (1980) preferred to describe the growth of some Collembola by two linear phases divided by an inflexion point between 4th and 5th instar. It can be argued that natural populations are not constrained to any theoretical growth models, but as long as the fit of data is reasonable and allows statistical comparisons, the choice of growth model is more a matter of finding the one with the best fit. Although von Bertalanffy's equation has been used to fit data for various organisms, severe limitations and disadvantages have been reported (Roff 1980 and references therein, Kaufmann 1981). Parts of these were overcome by transformation of data into a linear function describing the specific growth rate, which facilitated comparisons of effects of metals on growth. Differences were apparently subtle at moderately high concentrations of metals (Tab.4), and very large sample sizes would probably be required to demonstrate any significant effects on growth at a field site with moderate metal pollution. This is partly because of the comparatively low growth rate of O.armatus ($k < 0.040$, Fig.2; cf $k = 0.0133$ for O.furcifer reared at 15°C with a theoretical mean maximum length of 2.00 mm (Petersen 1971)). The lower threshold for lead in the F2-generation needs to be emphasized. The significantly reduced specific growth rate of O.armatus at 45 ppm lead in F2 compared to 150 ppm in F1 gives no evidence for an acclimation or adaptation to the contaminated environment. Unfortunately, the number of produced and hatched eggs by the F1 generation was too low for subsampling for lead analysis, and we cannot exclude the possibility that the lead concentration in the hatching eggs made a major contribution to a higher equilibrium concentration in the F2-generation.

The uptake and release curves of metals that are similar to those presented in this study for Onychiurus (Fig.1) have been reported for a number of invertebrate species and fishes (Smith et al. 1975, Brown 1977, Hartenstein et al. 1980). In terrestrial invertebrates uptake is likely to be an active process via absorption by the intestinal walls, but a major part of the metals become physiologically inactive due to different detoxifying mechanisms. In Collembola metals are stored to a great extent in epidermal cells of the intestinal walls (Humbert 1974, Joosse and Buker 1979). Two other detoxifying mechanisms observed in inverte-

brates, namely storage of copper in a lipid fraction (Wieser and Klima 1969) and synthesis of low molecular weight metal binding protein (Talbot and Magee 1978), may also be important in Collembola.

Since storage of metals in inactive states is no sufficient explanation for the metal release in Collembola, other mechanisms have to be considered. Joosse and Buker (1979) found that moulting was the major mechanism for a release process in Collembola. The effectiveness of this mechanism is probably age-dependent being higher at low ages, since immatures grow more rapidly and thus moult more often than adults do.

As Collembola require copper in respiration enzymes, an increased growth rate was expected on a copper enriched food (Fig. 2). The decline in growth rate at 15 ppm Cu may be due to coprophagy (cf Wieser 1978). At higher copper content in the food, the animals are able to extract sufficient amounts of the metal at the first ingestion, and as the food is rich in nutrients, the growth rate increases. Grazing of fungal colonies was also heavier at 45 ppm Cu and 15+15 ppm Pb + Cu than at 0 ppm and about twice as much mycelium was grazed after 30 days. When the food contains either too much copper for coprophagy or too little copper to justify only one ingestion, less energy and nutrients are used per unit time. Individual variations in assimilation efficiency are more important and the variation in length becomes substantial (Fig.2).

Reduced growth rate and theoretical mean maximum length at high metal contamination are of great importance for reproductive success. The length of O.armatus at the first egg-laying seemed to be relatively constant, 0.90 - 0.97 mm (unpubl.) except for the highest metal concentration, 150+150 ppm Pb + Cu, where the low k-value, which means a lower probability to survive to reproductive age, to some extent was compensated for by a smaller size of the animals at the beginning of the egg-producing period. However, that compensation was counteracted by delayed sexual maturity (unpubl.). The lowest metal concentration in the substrate at which a significant specific growth rate reduction occurred, 45+45 ppm Pb + Cu (Tab.4), gave an average equilibrium value of copper in Collembola of about 200 ppm (Fig.2). An average copper concentration around 175-200 ppm was found in O.armatus in soils within 650 m from the brass mill at Gusum, SE Sweden (unpubl.), where population density was reduced. Provided the up-

take mechanism for zinc, which is the predominating emitted metal at Gusum, is similar to that of copper and lead, the conditions may be close to those described for 45+45 ppm Pb + Cu. Though not lethal at the depths where O.armatus is abundant, the metal concentration in the vicinity of the Gusum mill may affect the growth and reproduction of the population. These long-term consequences of metal pollution may be even more significant for more edaphic species than O.armatus.

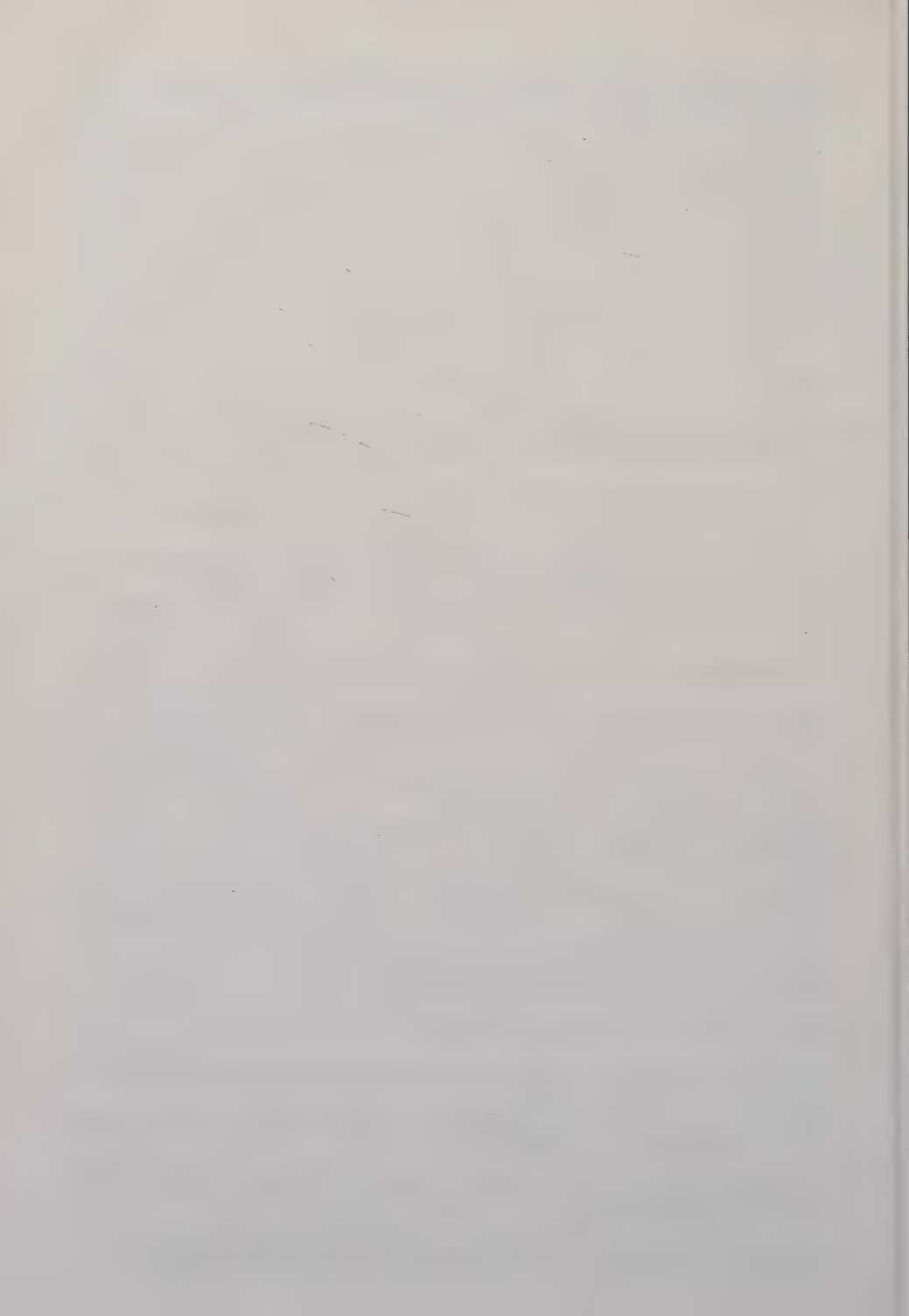
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POPULATION DENSITY AND TISSUE METAL CONCENTRATION OF

LUMBRICIDS IN FOREST SOILS NEAR A BRASS MILL

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ABSTRACT

The effect of metal pollution on species composition, population density, and age structure of earthworms was studied in coniferous forests near a brass mill in SE Sweden. Samples were taken in two biotopes, dominated by *Deschampsia flexuosa* and *Vaccinium myrtillus*, respectively, from eight sites at varying distances (175 - 20,000 m) from the mill. pH and CEC were found to increase in the soil close to the mill. A decrease in the concentration of extractable metals (Zn, Cu, Pb) could be followed up to 1.2 km from the emission source. Copper and lead were analyzed in pharynx, gizzard, muscles, clitellum, seminal vesicles and cerebral ganglion of different earthworm species. Generally, the concentration of copper in tissues was higher compared to lead and inversely proportional to the distance from the mill. A substantial metal uptake was found in vital tissues, such as seminal vesicles and cerebral ganglion. Juveniles tended to have higher metal concentrations compared to adults.

Density and biomass of earthworms was proportional to the distance from the mill, especially in the *Deschampsia*-dominated biotopes. *Dendrobaena octaedra* was the most abundant lumbricid species but was absent in soils within 1 km from the mill. Data indicate that the reduced density of earthworms near the mill was due to metal toxicity, but food deprivation could not be ruled out as a possible factor. Reasons for decreased density of earthworms at more remote sites are discussed and data on density and soil zinc concentrations are used to predict the density of earthworms at a metal polluted site.

INTRODUCTION

Scientists have long been concerned about the mutual benefits in the relation between earthworms and man (Darwin, 1881). During the 60's and 70's much attention was paid to the effects of environmental pollution on earthworms and the possible implications of these effects on man himself. Following the mercury debate in the mid-60's some research activity was focused on metal pollution of soils, and earthworms were sampled for metal analysis from selected environments such as roadside verges (Williamson & Evans, 1972, 1973; Gish & Christensen, 1973; Goldsmith & Scanlon, 1976; Ash & Lee, 1980) mines and industrial plants (Ireland, 1975, 1979; Ireland & Richards, 1977; Bull et al., 1977; Wright & Stringer, 1980), and sewage sludge treated soils (van Rhee, 1975; Andersen, 1979; Helmke et al., 1979; Hartenstein et al. 1980). Due to their ability to take up metals earthworms were suggested to be used for tests of soil contamination (Czarnowska & Jopkiewicz, 1978). Although some information has accumulated on metal concentrations in earthworms, little is still known about the ecological effects of sublethal levels of metal pollution.

The purpose of this study was to describe the relationship between metal pollution of a forest soil, tissue metal concentrations in earthworms, and population density.

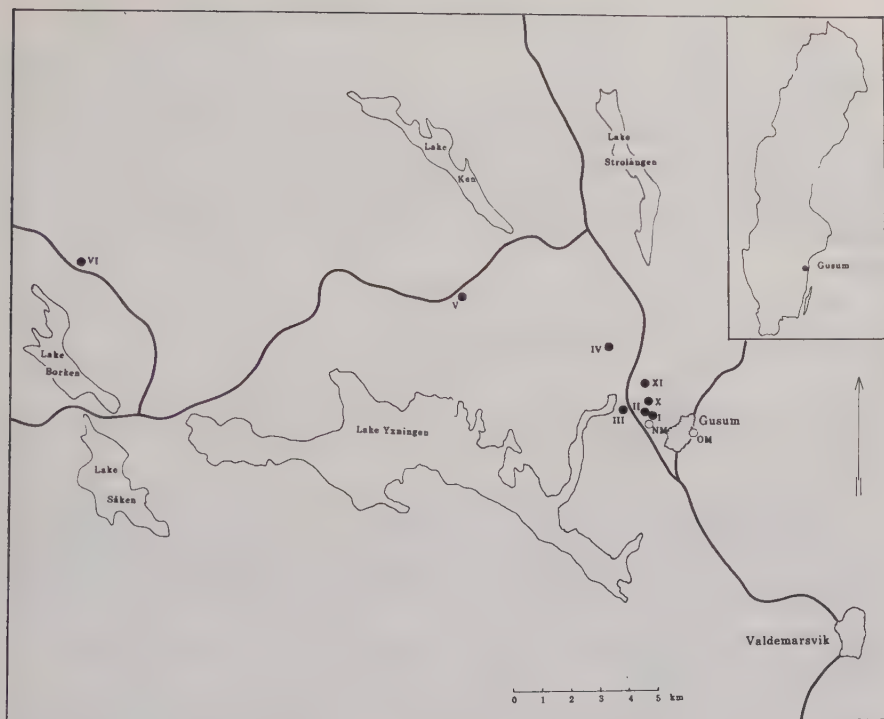


Fig. 1. Field study sites at Gusum, SE Sweden. The old brass mill is indicated by OM and the new brass mill by NM. Numbers of sites refer to the text.

STUDY AREA

The study was carried out near the village of Gusum, SE Sweden (Fig. 1), where a brass mill has been operating since 1661. The old mill in central Gusum was replaced in late 60's by a modern one, located approximately 1.5 km west of the village. This mill (smelter and foundry) is the only significant source of industrial pollution as well as the only important place of work in the region. Major elements emitted from the mill are zinc and copper (ca 98 % of emitted metals) and to a less degree lead and cadmium (Tyler, 1974).

Eight sampling sites at different distances from the mill were used (Fig. 1). One site (I) was close to the mill (175 m), while one of the control sites (VI) was about 20 km away. Five of the

sites (I-V) were chosen for a survey in 1978. Based on preliminary observations, the study was extended in 1979 to another three sites (VI, X, XI). The study sites were selected in mature (60-80 yr) spruce forests in the same NW quarter from the mill; the prevailing wind direction is SE. Most coniferous forests in the vicinity of the mill are only 20-40 years old and the selected sites are some of the few mature spruce forest islands in the area.

The sites were also used for sampling enchytreids, microarthropods, and large litter-active invertebrates. Some sites coincide with the sampling sites for macrofungi (Rühling, 1978), lichens and mosses (Folkesson, 1979), and microfungi (Söderström et al., unpubl.) and for studies of soil enzyme activity (Tyler, 1974, 1976).

Within each site two different biotopes were selected for separate studies. One biotope was mainly covered by Deschampsia flexuosa (L.) Trin. and the other by Vaccinium myrtillus L. The division was simply made to gain insights into the susceptibility of the soil animal community of two main biotopes of Scandinavian spruce forest areas.

SOIL CHEMISTRY

Soils for chemical analysis were sampled at each biotope and site from three depths, 0, 5, and 20 cm, equivalent to the A_0 , A_1/A_2 , and upper part of the B horizons. Soil samples were kept in a refrigerator at 2°C until analyzed.

$\text{pH}_{\text{H}_2\text{O}}$ (vol 1:1) of all samples was measured within 14 days. Ca, Mg, Na, and K were extracted from soils by continuous shaking in 1 M NH_4Ac (pH 7.0) for 2 hr. The extract was filtered and the filtrate evaporated to dryness. Residues were digested in nitric acid and perchloric acid, ratio 4:1. Na and K were analyzed by flame photometry and Ca and Mg by atomic absorption spectrophotometry (AAS).

Extractable metals (Zn, Cu, Pb) were extracted from the soil samples by the same method (1 M NH_4Ac ; pH 3.0) and analyzed by AAS.

EARTHWORM DENSITY AND BIOMASS

Earthworms were sampled in August 1978 (sites I-V) and in August 1979 (sites VI, X, XI) by the formaldehyde method (Raw, 1959). Fifteen squares (0.25 m^2) were randomly sampled in each biotope. Specimens were preserved in 80 % ethanol. The biomass of each square was determined by weighing the specimens after drying them one minute on a filter paper.

METALS IN EARTHWORM TISSUES

Lumbricids were sampled either by hand or by the formaldehyde method (site II). Specimens obtained by formaldehyde were immediately washed in water for at least 10 min. The worms were taken to the laboratory in plastic bags with soil from the sampling site and kept in a refrigerator for 2-3 days before species identification and age classification. The nomenclature follows Gerard (1964).

Before dissection the earthworms were allowed to empty part of the intestine for 24 hr on a filter paper in a plastic jar kept in a refrigerator (4°C). The following tissues were saved for analysis: pharynx, gizzard, muscles, clitellum, seminal vesicles, and cerebral ganglion. The intestine was omitted from analysis since we failed to get accurate replicates even after four days starvation of the specimens and a careful rinsing of the intestine walls with deionized water. Tissues were dried on watch-glass for two days at 85°C and weighed on a micro balance (Cahn 25). A micromethod was used (Bengtsson & Gunnarsson, 1982) to oxidize tissues by concentrated nitric acid and analyze copper and lead by flameless atomic absorption (Varian AA6). Zinc was not analyzed because of high blank values.

RESULTS

POPULATION DENSITY OF EARTHWORMS

Density and biomass of earthworms increased with increased distance to the mill (Fig. 2a, b) ($r = 0.9811$, $p < 0.01$ for densities in Deschampsia; $r = 0.8701$, $p < 0.01$ for densities in Vaccinium; $r = 0.9877$, $p < 0.01$ for biomass in Deschampsia; $r = 0.9206$, $p < 0.01$ for biomass in Vaccinium). Earthworms were generally

TABLE 1

Densities ($\text{ind} \cdot \text{m}^{-2}$) of adult/juvenile lumbricids at Gusum given for each species. Standard deviation ($n = 15$) for the mean of each population is shown in brackets. D = Deschampsia biotope, V = Vaccinium biotope.

Distance (m)	Site	Biotope	All species	Dendrobaena octaedra (Sav.)	Dendrobaena rubida (Sav.)	Lumbricus rubellus Hoffm.	Allolobophora rosea (Sav.)	Allolobophora caliginosa (Sav.)	Lumbricus terrestris L.	Octolasion cyaneum (Sav.)
175	I	D	0/0							
	I	V	0/0							
275	II	D	0/1.1					0/1.1 (2.8)		
	II	V	0/0							
650	X	D	0.3/0		0.3/0 (1.0)					
	X	V	0/0							
1,000	III	D	4.5/4.5	3.2/2.9 (4.0)				0.5/0.8 (2.0)	0.8/0.8 (2.0)	
	III	V	1.9/1.9	1.9/1.6 (3.4)					0/0.3 (1.0)	
1,250	XI	D	8.3/5.3	7.4/4.5 (9.6)	0.3/0 (1.0)	0/0.8 (1.7)	0.5/0 (1.4)			
	XI	V	7.7/14.4	7.7/13.1 (13.6)		0/1.3 (2.5)				
2,900	IV	D	8.8/8.0	5.6/5.1 (9.5)	0/0.5 (2.1)	2.1/1.3 (4.7)				1.1/1.1 (4.5)
	IV	V	1.9/1.3	1.9/1.3 (3.4)						
7,800	V	D	12.8/12.8	6.9/5.3 (6.2)	0.5/0.5 (3.2)	2.7/4.3 (8.1)		2.7/2.7 (8.0)		
	V	V	1.6/1.6	1.6/1.6 (5.2)						
20,000	VI	D	27.2/86.7	7.2/9.3 (11.6)	0.8/0.5 (4.2)	2.1/7.7 (6.6)	0.3/0 (1.0)	15.7/64.6 (64.8)	1.1/4.5 (6.0)	
	VI	V	15.2/43.7	1.6/1.0 (2.5)		1.4/4.2 (6.4)		9.9/28.5 (35.0)	2.4/9.9 (10.5)	

more abundant in the Deschampsia biotopes than in the Vaccinium biotopes ($p < 0.01$, two-way ANOVA). In both biotopes quite a few specimens were found in the vicinity of the mill (Table 1) in contrast to the extremely high and almost atypical numbers at the most remote site (Fig. 2). If data for site VI was omitted from the calculations of the correlation of density/biomass and distance, distance had no significant effect on neither density nor biomass in the Vaccinium biotopes ($r < 0.03$), while the correlation coefficient was still significant ($p < 0.01$) for the Deschampsia biotopes. Seven lumbricid species were represented in the samples but at most six (site VI) occurred at the same site. The most abundant species was Dencrobaena octaedra (Tab. 1), which usually occupies the litter and mor layers (Rundgren, 1975). However, the species was not found closer to the mill than 1 km. Lumbricus rubellus, often present in Swedish coniferous woods, was not obtained within the nearest 1.2 km, whereas Allolobophora caliginosa occurred at low densities near the mill (Tab. 1). The latter species contributed substantially to the abundance and biomass peak at site VI. At sites XI-V the proportion between adults and juveniles was even, whereas three to four times more juveniles than adults of the mineral-soil living species were obtained at site VI (Tab. 1).

SOIL pH AND CATION EXCHANGE CAPACITY

Could differences in population density and biomass be explained by differences in fundamental soil properties? Sites X-VI had a pH that is common in coniferous forest soils in that part of Sweden (Fig. 3), while a decreased acidity was found in the A_0 horizon (mor) close to the mill (sites I, II). When pH in the A_0 horizon was below 4.5, acidity was lower in the A_1/A_2 horizons. If sites I and II were omitted from a comparison because of the impact of the mill on the soil chemistry, pH values in the A_0 horizon of the Vaccinium biotopes were, except for sites XI and VI, below those in the corresponding horizon of the Deschampsia biotopes. The exceptions (site XI and VI) also support the observation that pH may be limiting for lumbricids in the Vaccinium soils. More individuals were found in the Vaccinium biotope than in the Deschampsia biotope only at Site XI (Fig. 2) ($p < 0.05$, t-test corrected for continuity). The density of lumbricids in the Vaccinium biotope at site VI was higher than the densities in

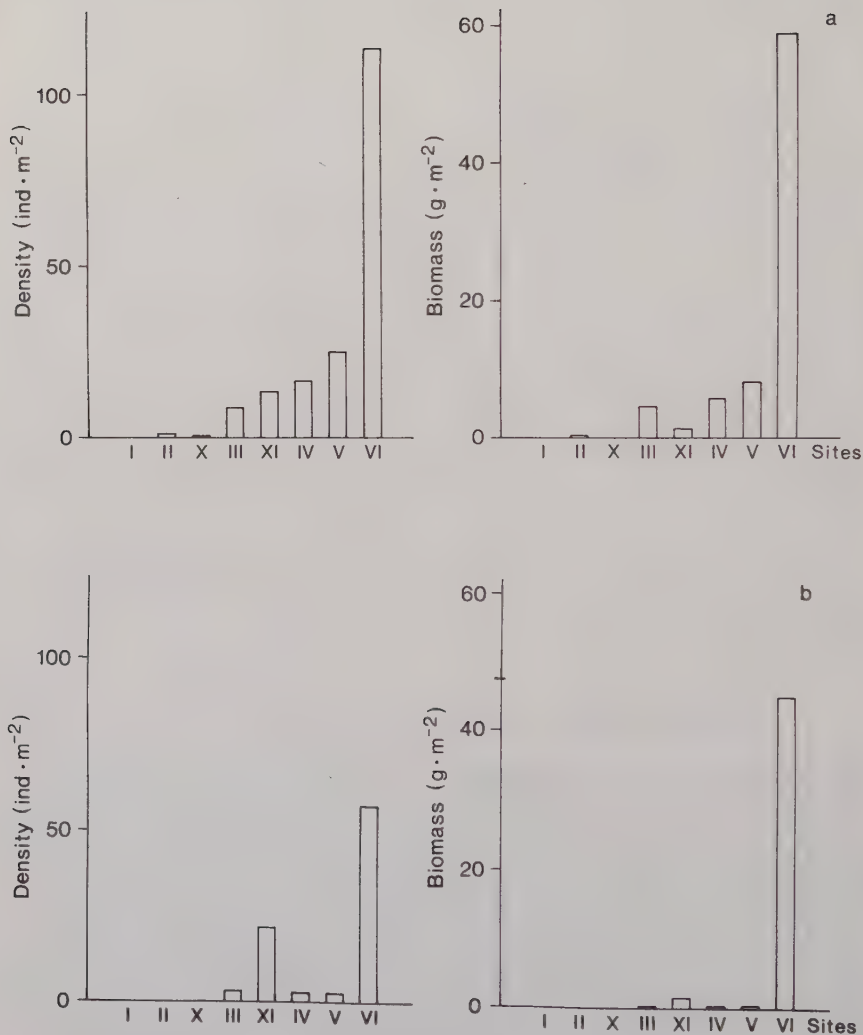


Fig. 2. Density (ind · m⁻²) and biomass (g w.w. · m⁻²) of earthworms at eight sites in the Gusum area. The sites are arranged according to their distance from the emission source.

a) *Deschampsia* biotopes, b) *Vaccinium* biotopes.

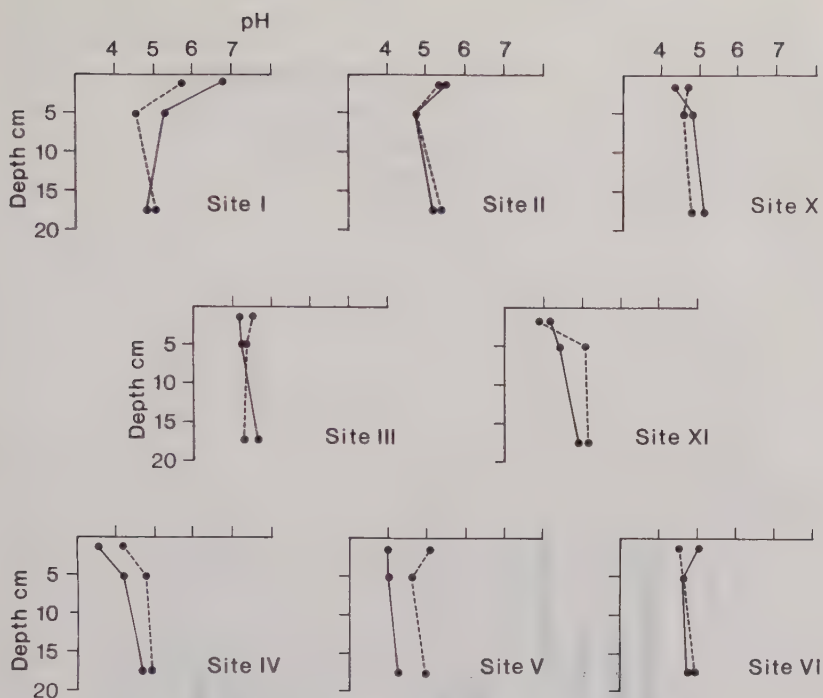


Fig. 3. Variation of $\text{pH}_{\text{H}_2\text{O}}$ with depth in soils of eight sites in the Gusum area.

- - - - = Deschampsia biotopes, — = Vaccinium biotopes.

all other Vaccinium biotopes together (Table 1) ($p < 0.05$, t-test corrected for continuity).

The cation exchange capacity was higher in the A_0 and A_1/A_2 horizons close to the mill (Fig. 4), where high concentrations of extractable calcium were demonstrated (Table 2). High calcium concentrations were also found at site III, which also had a comparatively high CEC, and at site X, i.e. the four sites closest to the mill. Exceptional low calcium concentrations were found at site XI, IV and VI. Concentrations of Mg, Na and K were broadly similar in all sampling sites of each biotope.

EXTRACTABLE METALS

Concentrations of lead, copper, and zinc were enhanced in the top soil up to 1.2 km from the mill (Tab. 3). The largest concentra-

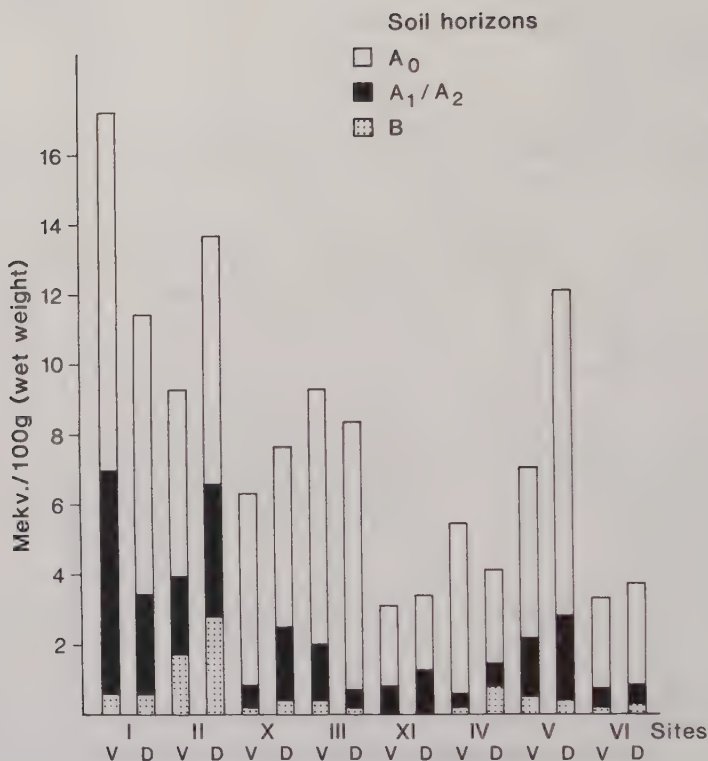


Fig. 4. The cation-exchange capacity ($\text{mekv} \cdot 100 \text{ g}^{-1}$) of studied soils in the Gusum area. D = Deschampsia biotopes, V = Vaccinium biotopes.

tions of metals occurred in the uppermost 4 cm (roughly the A₀ horizon) with less than one tenth (expressed in $\mu\text{mol/g d.w.}$) of these extractable metals leaching into the A₁/A₂ horizon. At depths exceeding 20 cm (B horizon) the amounts of extractable copper and lead were negligible, whereas high concentrations of zinc were found at these depths even at a distance of 1.2 km from the mill.

METALS IN EARTHWORMS

Copper concentrations in earthworm tissues were generally greater than lead concentrations, regardless of distance to the mill

TABLE 2

Concentrations ($\mu\text{mol} \cdot \text{g}^{-1}$ d.w.) of calcium in soils at Gusum. D = Deschampsia biotope, V = Vaccinium biotope.

Site	I	II	X	III	XI	IV	V	VI
Distance (m)	175	275	650	1,000	1,250	2,900	7,800	20,000
Biotope	D	D	D	D	D	D	D	D
	V	V	V	V	V	V	V	V
Ca								
A ₀	34.9	46.0	27.4	19.6	20.8	20.1	29.8	29.7
					7.3	7.1	6.1	14.1
A ₁ /A ₂	11.8	26.8	14.6	8.4	7.3	1.0	1.0	6.2
					4.3	2.1	1.4	0.2
B	2.1	1.8	11.4	6.6	0.9	0.2	0.1	1.3
							2.4	0.1
							1.0	1.5
							0.9	0.5

TABLE 3

Concentrations of extractable lead, copper, and zinc ($\mu\text{g} \cdot \text{g}^{-1}$ d.w.) in the A_0 , A_1/A_2 and upper part of B horizons in studied soils in the Gusum area. D = Deschampsia biotope, V = Vaccinium biotope.

Site	I		II		X		III		XI		IV		V		VI	
Distance (m)	175		275		650		1,000		1,250		2,900		7,800		20,000	
Biotope	D	V	D	V	D	V	D	V	D	V	D	V	D	V	D	V
Pb																
A_0	38.7	94.1	8.0	3.5	0.7	3.0	2.6	1.2	0.7	1.2	0.5	0.0	0.0	0.5	0.9	0.6
A_1/A_2	0.2	0.2	0.2	0.0	0.5	0.2	0.5	0.2	0.3	0.3	0.2	0.7	0.5	0.0	0.3	0.7
B	0.2	0.5	0.5	0.0	0.0	ND	0.0	0.5			0.2	ND	ND	0.2	0.3	0.6
Cu																
A_0	559.0	1688.0	92.6	14.6	2.6	4.2	1.1	2.6	0.6	0.6	0.2	0.1	0.2	0.2	0.1	0.1
A_1/A_2	0.6	2.6	2.0	0.2	0.6	0.6	0.4	0.3	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.2
B	0.6	0.5	0.6	0.7	0.3	0.1	0.2	0.2			0.2	0.2	0.2	0.1	0.1	0.1
Zn																
A_0	2168.0	2264.0	738.0	388.0	212.0	114.0	81.6	169.0	56.4	46.1	7.2	4.2	3.8	2.9	4.4	1.0
A_1/A_2	194.0	96.0	66.4	11.4	16.8	8.2	2.5	28.2	5.3	4.4	1.5	0.8	1.2	1.4	0.6	0.1
B	23.2	5.3	5.4	5.4	4.3	1.8	0.6	4.7			2.2	0.8	0.4	1.2	0.5	0.1

(Tab. 4, Fig. 5). Since the concentration of extractable lead in soils beyond site X, with few exceptions, was greater than the concentration of extractable copper (Tab. 3), this would indicate that earthworms like many other organisms have a specific demand for copper or, if not, that extractable amounts of metals in the soil are not an accurate estimate of metals available to earthworms.

The coefficient of variation for tissue metal concentrations was sometimes very high, close to 0.5 or higher, indicating a low precision of the analytical method or a true variation in natural populations. The most dramatic variation was found for pharynx and gizzard, both of which were exposed to soil that passed through the alimentary tract. Microscopic soil particles embedded in the tissues of the gut, may cause erroneous results.

The average lead concentration of tissues was not distance-dependent but seemed to vary unpredictably from one site to another and even to increase with increasing distance to the mill. Highest concentrations of lead were generally found in the pharynx, seminal vesicles and cerebral ganglion, and the concentrations were generally slightly higher in juveniles than in adults ($p < 0.1$, Mann-Whitney's test) (Tab. 4).

The pattern of copper distribution, one of the main pollutants from the mill, was more consistent. This was especially true for the two species and age groups dominating the samples, juvenile A. caliginosa and adult D. octaedra. The concentration of copper in the cerebral ganglion decreased for both categories as the distance to the emission source increased (Fig. 5) ($r = -0.6139$, $p < 0.05$ for A. caliginosa, $r = -0.4618$, $p < 0.1$ for D. octaedra). The concentration in muscle tissue decreased in A. caliginosa between site I and site V, but then increased at site VI. The copper concentration in the seminal vesicles of D. octaedra was higher close to the emission source than at more remote sites ($r = -0.6112$, $p < 0.05$) and conspicuously higher than in the seminal vesicles of other species studies. Concentrations of copper in the pharynx and to some extent in the gizzard increased slightly with increasing distance and were high in D. octaedra at site VI. Some of these differences were similar in the other species and age groups. However, it may be noted that the copper concentration in the seminal vesicles of adult A. caliginosa and L. rubellus increased with increasing distance from the mill.

The tissue concentration of copper was higher in juvenile

A. caliginosa compared to adults ($p < 0.01$, Mann-Whitney's test) while the reverse was true for D. octaedra. Adult D. octaedra had significantly greater concentrations in tissues compared to the other two species ($p < 0.01$, twoway ANOVA on log-transformed values).

PREDICTION

Can the data on earthworm density at Gusum be used to predict the density in a similarly metal polluted forest soil? To test within what limits the prediction can be justified, we calculated the regression of lumbricid density on the concentration of extractable zinc, which was the predominating metal in the mor. The observed $r = -0.6549$, d.f. 75, was statistically significant ($p < 0.01$). 95 % confidence limits were calculated for a) the average density of earthworms at Gusum, given a specific zinc concentration, b) the density in one single 0.25 m^2 square, given a zinc concentration, and c) the mean density of 15 random samples (0.25 m^2 square), given a zinc concentration (Fig. 6). Due to the very strong aggregation of earthworm populations, the prediction can not be made with very great precision. For example, at an extractable concentration of $20 \text{ } \mu\text{g/g}$ zinc in the mor, the predicted values for mean number of lumbricids per square meter falls between 8.4 and 22.0.

DISCUSSION

While several authors have reported uptake and accumulation of metals in earthworms (see Introduction), few have been concerned with the effects of metals on population densities and diversity. Williamson & Evans (1973), sampling earthworms by extracting soil cores in funnels, found no evidence for a decrease in densities of unspecified species in soils containing between 160 ppm and 8,000 ppm acid digestable lead. As their conclusion was based on a single soil core from each of 18 different localities, interpretations have to be made with care. Wright & Stringer (1980) were also unable to demonstrate any difference in population densities between a contaminated soil (pasture) and a control site (orchard). Presumably the two sites were not comparable, as they represented two different types of habitat.

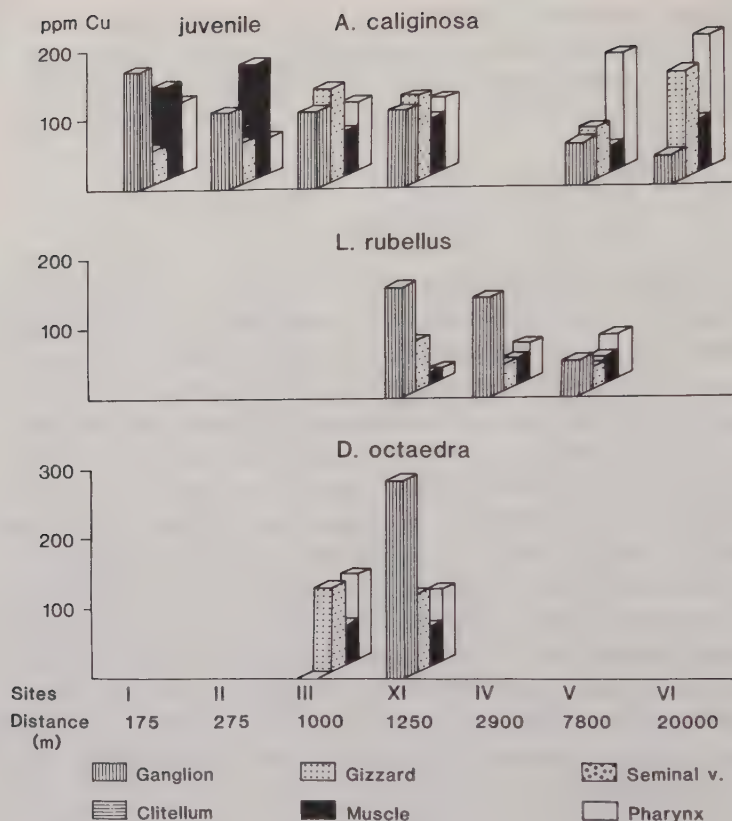
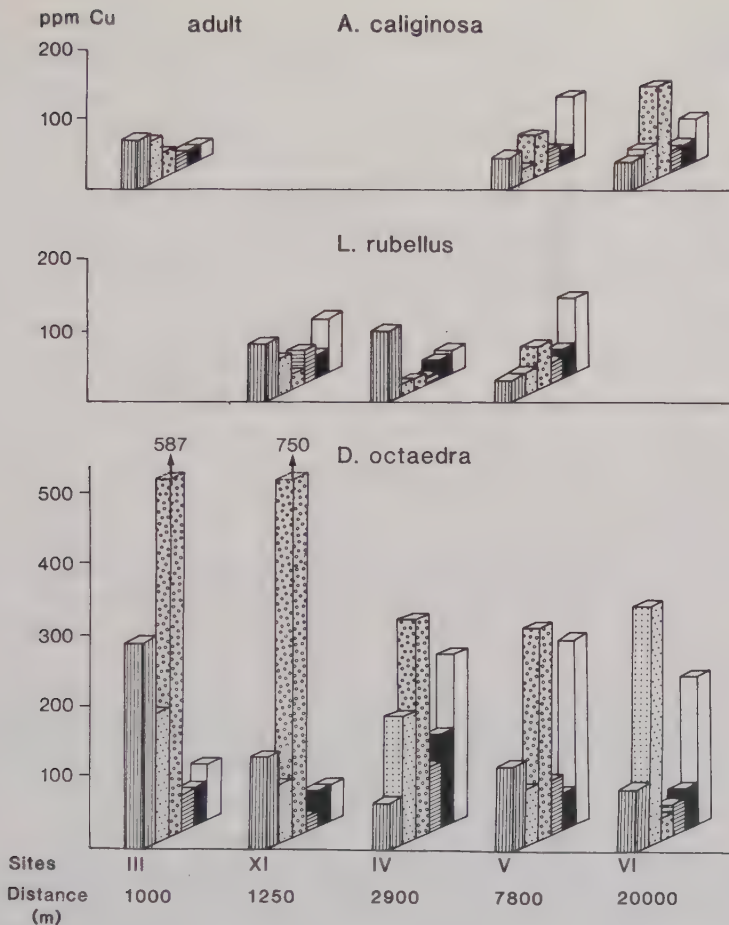


Fig. 5. Concentration of copper ($\mu\text{g} \cdot \text{g}^{-1}$ d.w.) in earthworms (adult and juvenile specimens) from sites in the Gusum area.

At least three possibilities may be suggested to explain differences in earthworm density and diversity at the mill at Gusum: (1) differences exist in soil properties, (2) metal concentrations are toxic, (3) food resources are deprived.

The two soil properties measured, pH and CEC, are in favour for a greater earthworm diversity close to the mill. Elevated pH, which may be due to deposition of amphoteric zinc oxide (Buchauer, 1973) should facilitate colonization by some acid intolerant species (Satchell, 1955), and increased CEC may indicate more efficient microbial decomposition of organic matter, providing suitable habitats for lumbricids. However, the increased CEC is almost entirely based on an increase of extractable calcium (Tab. 2) which indicates a specific pollution effect rather than supports



the assumption that the pool of extractable cations expands due to accumulation of organic matter and deficiency in root uptake. Two observations need to be emphasized. The first one is that the supply of extractable cations is not depleted by the metal pollution. The second observation concerns the high degree of similarity in soil chemistry between the sites, which justifies a comparison of metal pollution between them.

Since the mill is the only significant source of pollution in the area it is reasonable to claim, that metal emission is responsible for the reduced earthworm density and biomass (Fig. 2). The mode of metal action and the dynamics of metal - earthworm interactions still remain unclear. Copper seems to be the most detri-

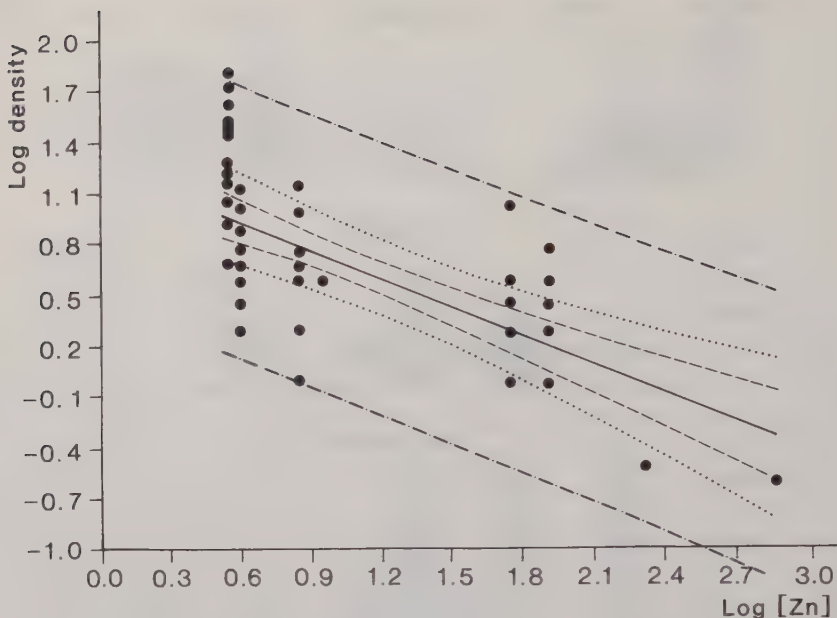


Fig. 6. Regression of log density (ind $\cdot$ 0.25 m⁻²) of earthworms on log concentration of extractable zinc ($\mu\text{g} \cdot \text{g}^{-1}$) in the mor (A₀ horizon) for data from the Gusum area. Equation for the regression line is $y = 0.1274 - 0.556x$.

Symbols for 95 % confidence intervals:

----- = average density of earthworms at Gusum,

- - - - - = density in one new 0.25 m² square,

..... = mean density of 15 randomly sampled 0.25 m² squares.

mental of the metals to the worms. Lead tends to be tightly bound to humus particles in the soil and the ratio of lead in earthworm tissues versus soil is usually far below unity (Hartenstein et al., 1980; Wright & Stringer, 1980). The minor influence of elevated soil lead concentrations on tissue uptake (Tab. 4) may also be accounted for by other circumstances. Thus, absorption of lead by earthworms is reduced in the presence of high dietary concentration of copper (Petering, 1974) and high concentrations of calcium in soils may depress lead uptake (Ireland, 1979 and references therein). Earthworms are known to be sensitive to copper (van Rhee, 1963, 1967) and *Eisenia foetida* (Sav.) was found to suffer from 100 % mortality in sewage sludge enriched with 2,500 ppm copper during a 1-week experiment (Hartenstein et al., 1980). Although the concentration of extractable copper in soils close

to the Gusum mill is less than 2,500 ppm (Tab. 3), the limit for 100 % mortality at Gusum may be lower due to a simultaneous uptake of other metals. Furthermore, it has to be remembered that the proportion of metals in a soil available to earthworms has not yet been properly defined.

We used the data on concentration of extractable zinc rather than copper for prediction of earthworm densities (Fig. 6), since this was the predominating metal in the soil and our scattered data on zinc in earthworm tissues also indicate an extensive uptake of this metal. Essentially the same ambiguous result was achieved when regression was made on the sum of zinc and copper in litter, but for obvious reasons the regression line was steeper (unpubl.). The method can be considerably improved if the number of sampled squares is increased to e.g. 50, since this will decrease the standard error. However, given narrower confidence intervals, less variation in other soil parameters essential to earthworms is assumed for a precise prediction. To test this presumption data from more field sites have to be collected. Meanwhile, the importance of making predictions of earthworm densities should be evaluated; principally, on a more subtle level, we want to know how many trees one hundred earthworms can raise.

The disproportional age structure (Tab. 1) supports the lethal toxicity concept. Table 1 implies a higher juvenile mortality rate (or a reduced fertility) at the most polluted sites and where comparisons can be made, juveniles tend to have higher copper concentrations in tissues than adults (Fig. 5). The individual variation is substantial (Tab. 4) and it may be assumed that the juveniles with low metal concentrations have a greater chance to become adults and also are responsible for the comparatively lower metal concentrations in adult tissues compared to juvenile tissues.

There are some evidence (Satchell, 1967; Parle, 1963a, b; Bolton & Philipppson, 1976; Pearce, 1978; Cooke & Luxton, 1980) that microorganisms are essential as a food source for at least some earthworm species. No information is available on the distribution of bacteria at Gusum, but the length of active fungal mycelium is greatly reduced within 500 m from the mill (Nordgren, pers. comm.). Since bacteria in general show about the same range of tolerance to soil zinc and copper pollution as fungi (Jordan & Lechevalier, 1975), the soils close to the mill are probably deprived of microorganisms and food shortage cannot be eliminated

as a hypothesis for reduced earthworm density. Too little is, surprisingly, known about earthworm food selection to permit any definite conclusions until more experimental work has been carried out.

Species differences in density (Tab. 1) and tissue metal concentrations (Fig. 5) may well be explained by differences in feeding habits. Whereas D. octaedra is a litter-dwelling species, A. caliginosa and L. terrestris also occupy the mineral soil (B horizon). L. rubellus usually occupies the intermediate depth (Nordström & Rundgren, 1973). Although D. octaedra did not occur in the most stressed sites, it was found at sites where the densities of such coniferous soil species as D. rubida and L. rubellus were still low (Tab. 1). Probably this can be accounted for by the high reproductive output of D. octaedra. It should be observed that D. octaedra was entirely absent within 650 m from the mill, while a few juvenile A. caliginosa were sampled even at 275 m distance. Since especially litter and to a certain degree humus are the principal metal sinks in a forest floor, deep burrowing species may be less affected by deterioration of the microbial community and less likely to accumulate metals from microorganisms and/or detritus.

To explain the lower earthworm density at sites XI, III, and IV, where letal metal concentrations or significantly decreased microbial biomass (Nordgren, pers. comm.) do not seem to exist, additional information has to be taken into account. Metals are not evenly distributed between tissues but seem to have a high affinity to cerebral ganglion and seminal vesicles (Fig. 5). These tissues are probably rich in protein with excess metal binding groups such as sulphhydryl and carboxyl. Other sites of metal concentration have been proposed. Ireland & Richards (1977) found deposits of lead in the chloragosomes in the posterior alimentary tract and suggested interferences with glycogen metabolism in the absence of a diet rich in energy. Wielgus-Serafińska (1979) found elevated lead levels in the cytoplasm and vacuoles of the epithelial gland cells and in the sarcoplasm of the muscle cells in the body wall. Disturbances of the Ca-transport in the sarcoplasmic reticulum were assumed to be responsible for the decreased muscle activity of experimental earthworms.

Since we decided not to analyze metals in the intestines because of irreproducible results, we have no data to discuss the

importance of metal deposits in the alimentary tract. But we want to emphasize the importance of separate analyses of earthworm tissues to reveal metal influences on behaviour. The evidence for concentration of metals to cerebral ganglion and sarcoplasm strongly suggests possible disturbance of burrowing capacity. Previous work (unpubl.) has shown a limited burrowing capacity in Allolobophora longa Ude in lead contaminated soils. Reduced burrowing would imply less probability for the earthworms to escape from drought or predators and/or to find food and this would ultimately lead to less mixing of the soil.

A concentration of metals to the seminal vesicles, like that found in D. octaedra might have profound effects on reproduction but so far, no data have been published on the reproductive output. Inability to reproduce normally and to burrow efficiently may be important reasons for the decrease in density beyond sites I and II at Gusum, i.e. at sites with sublethal amounts of extractable metals. If this is true, much more effort should be devoted to research aimed at finding limits of metal pollution, that significantly affect the behaviour of invertebrates which in turn has long-term consequences for the decomposition processes in the soil.

ACKNOWLEDGEMENTS

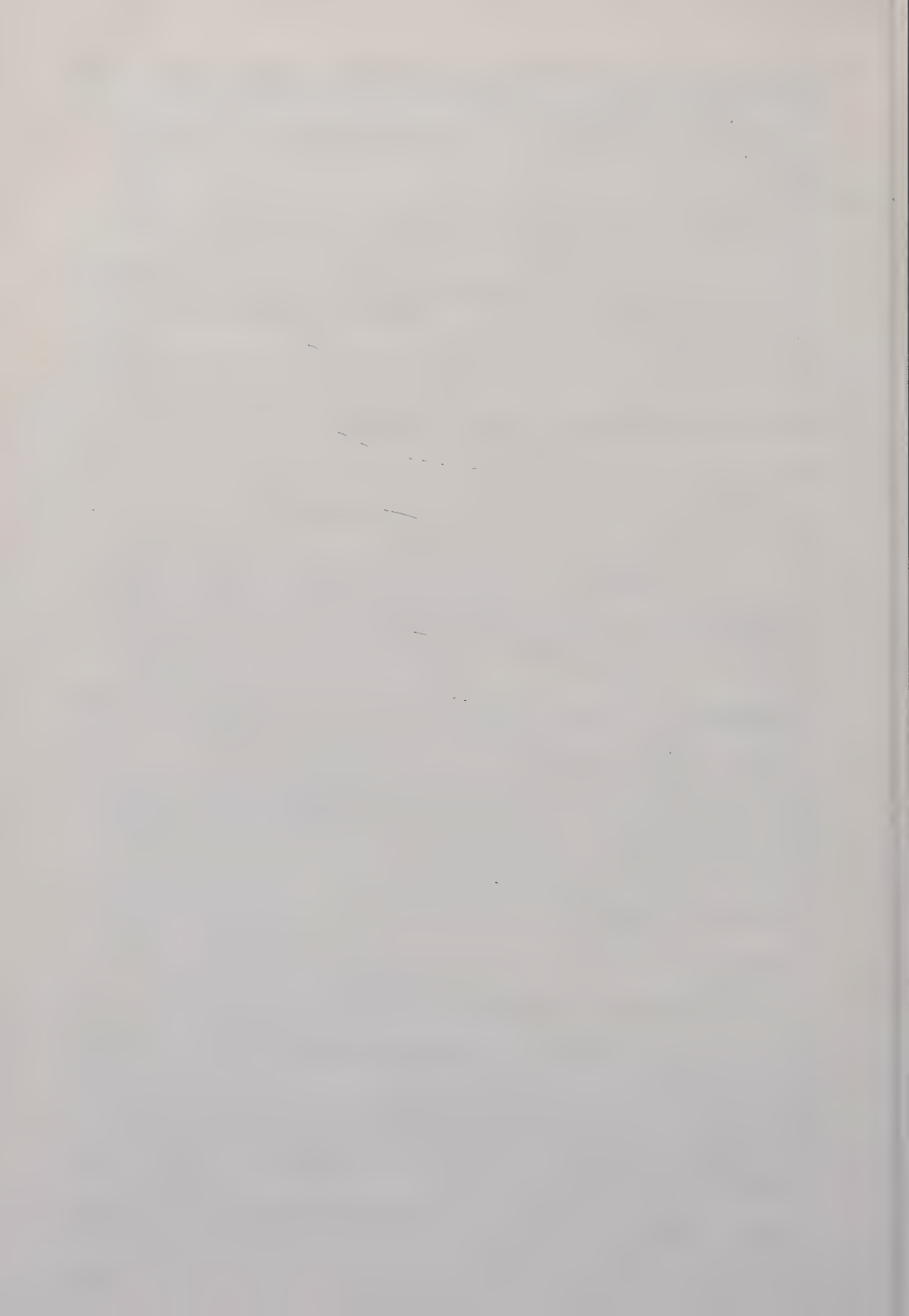
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RESPIRATION AND GROWTH OF A FUNGUS, MORTIERELLA ISABELLINA

IN RESPONSE TO GRAZING BY ONYCHIURUS ARMATUS (COLLEMBOLA)

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Colonies of the fungus Mortierella isabellina (Oudem) were grazed by the Collembola Onychiurus armatus (Tullb.) for short periods interrupted by incubation without grazing in laboratory experiments. The grazed fungus had a lower respiration than ungrazed when Collembola were present on the mycelium. Fungal respiration was significantly increased with grazing when Collembola were periodically removed from the mycelium. Metal contamination of the substrate increased fungal respiration, regardless of grazing. Length of fungal mycelium was significantly greater in a natural soil in the presence of Collembola but reduced in a metal polluted soil. Factors that may determine the impact of grazing on fungi are discussed.

INTRODUCTION

The role of soil invertebrates in decomposition processes is still poorly understood despite extensive work in the 70's under the International Biological Programme. For many years scientists have argued that soil invertebrates increase the release of nutrients by fragmenting litter, by improving soil structure and by grazing upon microorganisms. The extent to which enhanced microbial activity in a soil is affected by grazing microarthropods is controversial, largely because of the inadequate knowledge of the food specialization of microarthropods. One view holds that Collembola have developed a generalist's feeding strategy in association with an excess of available food (Anderson and Healey 1972), while another view is that they may be generalists during periods of shortage of particular food items but in times of affluence show preferences for specific foods.

Evidence from laboratory experiments indicate that most collembolans prefer feeding on fungi (Singh 1969, McMillan 1975) and under certain circumstances seem to behave as catalysts of fungal activity and growth by dispersing spores, by eliminating mycostasis and by grazing (Wiggins and Curl 1979, Hanlon and Anderson 1979).

The objectives of the present study were to test, firstly whether grazing by a collembolan species, Onychiurus armatus (Tullb.) could be eliminated as an explanation for enhanced activity of the fungus Mortierella isabellina (Oudem), secondly whether these collembolans stimulated or inhibited the growth of the fungus in a natural soil, and thirdly whether collembolan influence on fungal growth was modified in a metal contaminated soil. Both species are common in coniferous forest soil in South-east Sweden. The experiments were designed to stimulate grazing on patchily distributed fungal mycelium.

MATERIAL AND METHODS

RESPIRATION EXPERIMENT

Growth vial: A small plastic cup ($\varnothing$ 15 mm, depth 6 mm) was placed on the bottom of a 26 ml vial. A mixture of plaster of Paris and activated carbon (9:1, w/w) was dispersed around the cup and left to solidify. The plastic cup was removed and the vial sterilized at 120°C for at least three hrs. Another plastic cup, sterilized with UV light in a sterile cabinet for 25 hrs, was fitted into the cavity on the bottom of the vial. An agar solution was poured into the cup.

Fourteen vials were prepared with pure agar solution in the cups (2 % Bacto agar, 2 % Oxoid malt extract and 30 ppm chlortetracycline), and another fourteen vials were provided with an agar-metal solution, the final concentration of which was 150 ppm Pb and 1500 ppm Zn.

A piece of sterilized nylon net (Nytal, 62 μ m) covered the plastic cup just above the agar surface and was attached to the plaster of Paris by a string of palmitic acid. This ensured that the agar could not be reached by grazing Collembola. The plaster of Paris was saturated with sterilized distilled water. A spore suspension of a stock culture of M.isabellina was inoculated aseptically on the agar through the nylon net. The vials were sealed with a rubber septum and incubated at 15°C until half of the plastic cup was covered with mycelium.

Stock culture of Collembola: O.armatus were extracted from the mor of a spruce forest soil, 20 km W of Gusum, SE Sweden, by a modified Macfadyen extractor. They were maintained on a diet of M.isabellina growing on agar in three small plastic cups immersed in plaster of Paris and activated carbon in a 50 mm Petri dish, that was further immersed in plaster of Paris and activated carbon in the middle of a 150 mm glass Petri dish. The 50 mm plastic Petri dish was removable and regularly exchanged when most of the mycelium had been consumed. Contamination of the fungi by dead Collembola was avoided by use of three separate cups with small batches of mycelium; the collembolans were seldom trapped in the mycelium, which happened frequently in preliminary studies when wider cups were used for mycelial growth.

Test procedure: The experiment was aimed at stimulating the grazing pressure on a patchily distributed mycelium. A patch was defined as a colony in a vial, and variation in grazing intensity

on the colony was achieved by regular supply and removal of animals. A patch was grazed for a two day grazing period, then ungrazed for five days (grazing interval), grazed for two days, and so on. We have observed a similar feeding behaviour frequently in our stock culture of Collembola, where a large portion of the population may be engaged in other activities than feeding, and where one or two out of three colonies of mycelium are periodically ungrazed. The apparent effect on the fungus is a period of growth unlimited by grazing. The hypothesis we wanted to test was that periodical grazing of mycelium resulted in decreased respiration from the mycelium, regardless of metal contamination in the substrate.

Onychiurus were randomly picked from the stock culture and added to vials in the following combinations (seven replicates per combination):

- pure agar + 5 Onychiurus
- pure agar + 0 Onychiurus
- metal-agar + 5 Onychiurus
- metal-agar + 0 Onychiurus

Each vial was purged for one min with filter sterilized compressed air and sealed. A 0.2 ml air sample was taken through the rubber septum by a syringe and analyzed for CO₂. The vials were incubated at 15°C.

After 48 hrs a 0.2 ml air sample was analyzed for CO₂. The collembolans were gently removed from the vials and all vials were purged as above. A 0.2 ml air sample was analyzed and the vials were incubated at 15°C for five days. Another 0.2 ml sample was then analyzed for CO₂. Collembola were randomly picked from the stock culture and added to the same fourteen vials. All vials were purged, resealed and incubated for another 48 hrs grazing period. The procedure above was repeated four times until the fungal cultures had grown over the agar surface and part of the colonies had become senescent. The entire experiment was repeated twice.

CO₂ analysis: A Varian model 3700 gas chromatograph equipped with a thermal conductivity detector (TCD) was used for the determination of CO₂. Separations were performed on a 3 m x 1.8" o.d. stainless steel column packed with Porapak Q, 80/100 mesh at 30°C. Helium was used as a carrier gas (25 ml min⁻¹) and the filament was held at 300°C.

FUNGAL GROWTH EXPERIMENT

Test procedure: To make sure that a positive or negative effect of grazing on fungal respiration in the respiration experiment corresponded to a similar change in net productivity, measured as fungal growth, a second experiment was done in a natural soil. Our hypothesis was that presence of O.armatus in a soil would decrease fungal standing crop, regardless of metal contamination in the substrate.

Test procedure: Test tubes ($\varnothing$ 20 mm, 20 cm length) were filled with mor soil to 20 mm depth. The soil was taken from 0-2 cm depth at two sites, 275 and 7,800 m from a brass mill at Gusum, SE Sweden (sites II and V in Bengtsson, Nordström, Rundgren, in press).

The metal concentrations in the soil were different at the two sites. 1300 ppm Zn and 200 ppm Cu were found close to the mill and 30 ppm Zn and 9 ppm Cu at site V. Eighty test tubes were used with soil from each of the sites, capped with a cotton plug and autoclaved at 120°C for one hr. A small amount of sterilized distilled water was added to the soil, and the tubes were incubated at 20°C for one week prior to the experiment. The test tubes were kept in moistened, sealed plastic bags during incubation. Each tube was then inoculated with 0.25 ml of a spore suspension of M.isabellina and incubated for one week at 20°C. Patchiness of fungal hyphae was easily recognized by visual inspection of the soil surface in the tube.

Length of fungal mycelium at the start of the experiment was determined in ten test tubes, five from each soil. Plate counts for bacteria were made on a subsample from each tube. Onychiurus were randomly picked from the stock culture and added to the test tubes in the following combinations:

- 40 tubes unpolluted soil + 6 Onychiurus per tube
- 35 tubes unpolluted soil + 0 Onychiurus
- 40 tubes metal-polluted soil + 6 Onychiurus per tube
- 35 tubes metal-polluted soil + 0 Onychiurus

The abundance of Collembola in tubes corresponded to a density of 19,000 ind m^{-2} , a rather low figure for Collembola in unpolluted coniferous forest soils in southern Scandinavia (Persson and Lohm 1977, Bengtsson and Rundgren unpubl.). However, on a volume basis the abundance in the test tubes was higher than that found in a natural mor.

During a seven week incubation, 20 test tubes (five of each combination) were sampled weekly and analyzed for length of

fungus mycelium and number of bacterial colonies. After seven weeks the remaining ten test tubes were examined for the presence of Collembola. The experiment was repeated twice.

Length of fungal mycelium: Hyphal length of active mycelium was determined by a vital staining technique (Söderström 1977). Samples of soil from 3-6 g each were homogenized in 100 ml phosphate buffer (60 mM, pH 7.5) for 1.5 min by a Sorvall^R Omni-Mixer at 8,000 rev min⁻¹. The suspension was diluted 500 times and fluoreseindiactate (FDA, 10 µg ml⁻¹) was added. The hyphae were stained for three min and then collected on a 22 mm membrane filter (0.8 µm Millipore). Mycelial length was measured with the intersection method at 480 x magnification.

Bacterial colonies: Subsamples of soils were taken from the test tubes for determination of bacterial contamination. The samples were homogenized, diluted and spread on plates of 1.5 % tryptone-soy-agar supplemented with 70 ppm Cycloheximide to suppress fungal growth.

RESULTS

The respiration was much higher after the first grazing interval than after the latter part of the experiment (Fig.1). Fungal respiration was initially lower on metal contaminated substrate and also lower in vials with mycelium grazed by Onychiurus. The respiration was higher on metal contaminated substrate and from grazed mycelium after the first interval between grazing periods. The respiration rate was significantly different in grazed compared with ungrazed and in contaminated compared with uncontaminated colonies (Fig.1). As the agar became fully colonized and the cultures senescent, the fungal respiration declined to a much lower level than during the initial rapid phase.

While grazed mycelium respired more than ungrazed after the animals had been removed, grazing suppressed respiration of fungi while Collembola were present in the vials (Tab.1). Differences in respiration after the grazing periods disappeared at the end of the experiment when the substrate was fully colonized. Except for the first grazing period, respiration from contaminated mycelium exceeded respiration from uncontaminated mycelium.

Hyphal length was significantly greater in grazed samples than in ungrazed, regardless of soil type (Fig.2). The length of active mycelium declined slightly in the absence of Collembola and

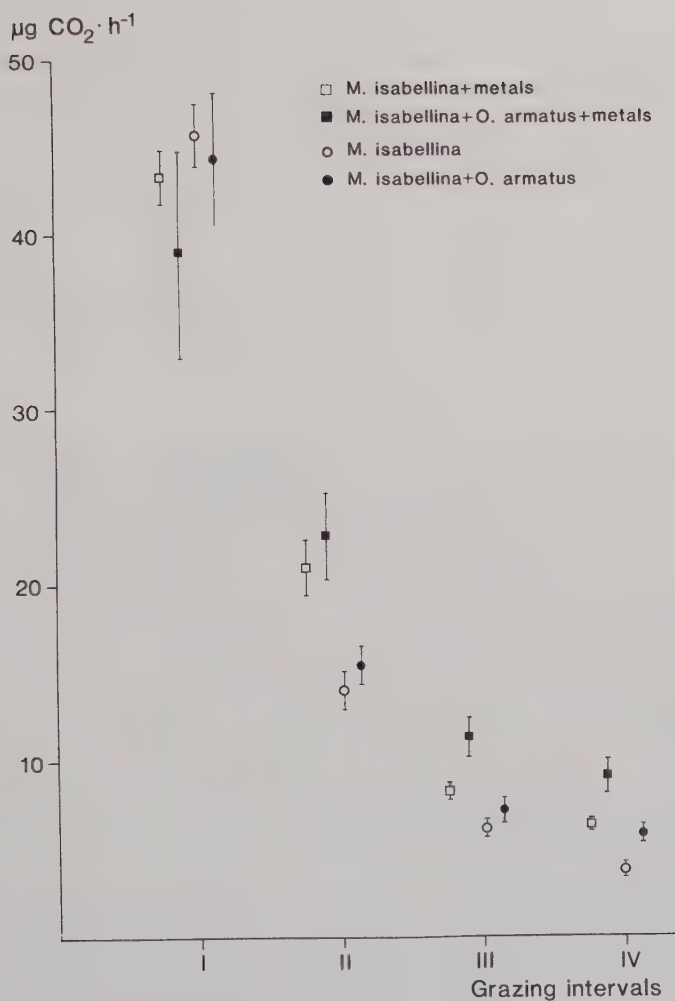


Fig.1. Respiration ($\mu\text{g CO}_2 \text{ hr}^{-1}$) of colonies of *Mortierella isabellina* after 5 days incubation. The incubation was repeated 4 times. Mean and standard error are given. Respiration was significantly different between grazed and ungrazed systems (two-way ANOVA, $F = 6.43$, $p < 0.05$ in uncontaminated systems; $F = 10.90$, $p < 0.01$ in contaminated systems) and between metal contaminated and uncontaminated systems ($F = 36.93$, $p < 0.0005$ in grazed systems; $F = 28.32$, $p < 0.002$ in ungrazed systems).

TABLE 1

Respiration of *Mortierella isabellina* after a two day grazing period in grazed (g) and ungrazed (ug) vials with metal contaminated (c) and uncontaminated (uc) substrate. The grazing periods were separated by a five day grazing interval with *Collembola* removed from the mycelium. Mean ($\mu\text{g CO}_2/\text{ml}$) and standard error ($n=7$) are shown for respiration, corrected for respiration by *O. armatus* (average of $7 \mu\text{g CO}_2/\text{ml}$).

Grazing period	I	II	III	IV
g + uc	127.0 $\pm$ 5.1	78.0 $\pm$ 7.6	30.2 $\pm$ 2.7	9.9 $\pm$ 1.7
ug + uc	135.2 $\pm$ 3.4	89.0 $\pm$ 7.7	33.2 $\pm$ 2.2	10.7 $\pm$ 1.5
g + c	68.2 $\pm$ 7.9	106.6 $\pm$ 9.7	38.7 $\pm$ 5.1	18.4 $\pm$ 1.6
ug + c	83.3 $\pm$ 9.4	118.3 $\pm$ 3.6	46.8 $\pm$ 1.2	18.0 $\pm$ 1.9

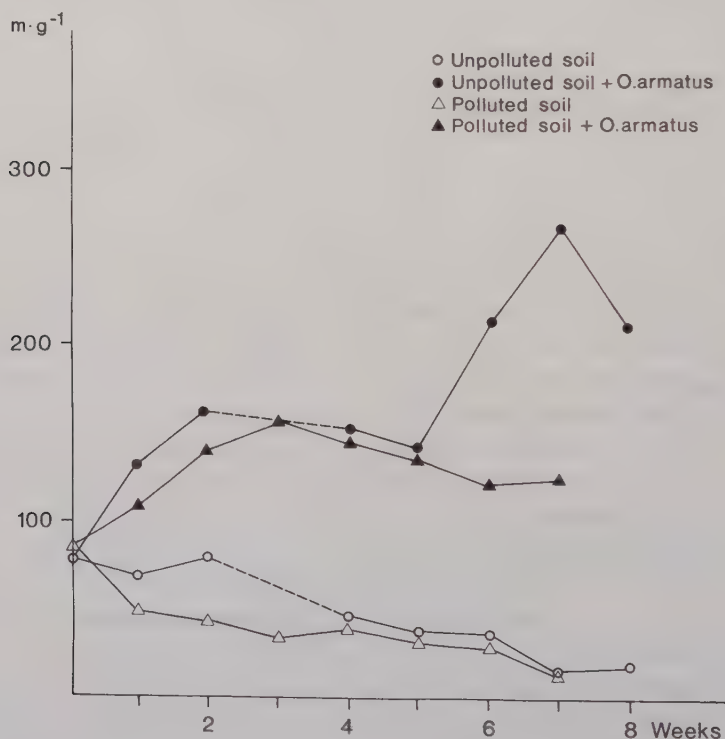


Fig.2. Length of fungal mycelium (m.g^{-1} soil) in two kinds of soils (metal polluted and unpolluted mor; 1,300 ppm Zn and 200 ppm Cu, 30 ppm Zn and 9 ppm Cu, respectively) with or without *Collembola*. Grazing increased length significantly in both kinds of soil ($p < 0.00005$, two-way ANOVA). Mycelial length was significantly lower in metal polluted soil than in unpolluted in presence of *Collembola* ($p < 0.025$). However, during the first 5 weeks incubation, this difference was insignificant ($p > 0.05$).

in presence of Collembola in polluted soils after the initial burst of growth. The hyphal length was significantly different in the two kinds of soil in the presence of Collembola, only during the last three weeks of the experiment (Fig.2). Hyphal lengths in the polluted and the unpolluted soil, were not significantly different in the absence of animals (Fig.2).

Bacteria were present in low numbers when Onychiurus was present. After incubation for one week an average of 240 colonies per gram soil were found and after seven weeks the average was five. Bacteria were probably introduced by the animals.

An average of 4.5 Collembola were recovered from the soil samples at the end of the experiment.

DISCUSSION

Although several reports have attributed the decline of a fungal standing crop and respiratory activity in a sterilized system to grazing by Collembola (van der Drift and Jansen 1977, Hanlon and Anderson 1979, Parkinson et al. 1979, Hanlon 1981) we were unable to reproduce those effects in our experiments. Colonies of M.isa-bellina that were periodically grazed by O.armatus responded to grazing with a significantly higher respiration than ungrazed colonies. The respiration increase corresponded to a higher net productivity, measured as fungal growth, in sterilized soils.

What insights can be gained about the influence of Collembola on fungi from the differences in design and outcome of these and other experiments reported in the literature?

a) The importance of patchiness of fungal mycelium for the effect of grazing Collembola should be emphasized in an experimental design. Little is known about microdistribution of fungi in the soil and we have no direct evidence for patchiness. Indirect evidence, however, is circumstantial. Microsampling of soil fungi is laborious for statistical reasons (Bååth and Söderström in press), and in laboratory experiments aggregation of fungal hyphae can be observed, e.g. in connection with large pores in the soil.

Models for foraging in a patchy environment propose that the forager remains in a patch until a fixed amount of the resource has been used, or remains in a patch for a fixed period of time, or remains in a patch until the amount of available food falls below a certain value (Krebs 1973). Clearly, the first two strategies

are not efficient when patches vary in density. Laboratory observations (unpubl.) show that foraging O.armatus remain on dense, young and sporulating patches of fungi for a fixed period of time, but when the patches vary in size, density and senescence, Collembola stay longer on dense mycelium with a low degree of senescence compared to mycelium with a larger proportion of senescent hyphae. The intensity of grazing on a certain patch should then vary from time to time, depending on such factors as species composition in the patch and density of Collembola. In our respiration experiment Collembola remained in the artificial patch for a fixed period of time, while in the growth experiment we do not know exactly what strategy they had in foraging of mycelial patches. Provided that the differences in respiration from grazed and ungrazed colonies during the two day grazing period (Tab.1) were representative for another five to ten days grazing, the importance of simulations of patchily distributed mycelium in grazing experiments with Collembola is unequivocal. While periodical removal of O.armatus from mycelium resulted in increased respiration of grazed compared to ungrazed systems, the natural heterogeneity in the soil microhabitat provided the necessary conditions for a patchiness of mycelial distribution and for a positive effect of grazing on fungal standing crop.

b) If Collembola show a food specialization, seasonal or not, the choice of fungal species for an experiment may be critical. Our studies on the food preference of O.armatus (Bengtsson et al. in press) showed that some fungal species were grazed more than others and that the most preferred fungi also induced a greater egg production. However, several fungal species are actively fed upon and may be found in the gut of O.armatus (Singh 1969). It is not known whether the relative abundance of fungal species is the same in the soil as in the gut of Collembola in a mixed species environment, but we assume that the preferred fungal species are more actively searched for than less preferred species in a few species environment. Since M.isabellina was not one of the most preferred fungi in our preference experiment, it was anticipated that the search image of O.armatus would not eliminate the fungus in a single species grazing experiment in soil. In experiments with Folsomia candida (Willem.) Hanlon and Anderson (1979) used Coriolus versicolor (L. ex Fr.) and Hanlon (1981) used Botrytis cinerea (Pers. ex Fr.) to show reduced oxygen consumption by the fungi after grazing. Parkinson et al. (1979) used two other

fungus species, viz. Basidiomycete 290 and Sterile dark 298, isolated from the litter layer in an aspen forest. While Sterile dark 298 was one of the most preferred species by O.subtenuis, the Basidiomycete form was generally avoided (Visser and Whittaker 1977). These two species showed evidence for interspecific competition; while Sterile dark 298 was heavily fed upon by Collembola and declined in standing crop, Basidiomycete 290 was toxic to the animals. van der Drift and Jansen (1977) used the indigenous microflora of isopod pellets for a grazing experiment and reported an increased respiration rate and reduced hyphal density due to grazing, but their estimate of fungal density was uncertain.

c) As outlined by Petersen (1980) there may be some general differences in reproductive behaviour and metabolic rate between those Collembola species living at the soil surface and those living in the interstitial pores. There may also be certain differences in behaviour between Collembola species in the same habitat - some are very active and migrate daily, while others may occupy the same pore space for a long time. The difference in growth and metabolism of fungi due to grazing by Collembola may be a consequence of different feeding behaviour of the grazer. For example, F.candida studied by Hanlon and Anderson (1979) and Hanlon (1981) is, at least in laboratory experiments, a more active searcher and grazer than O.armatus. F.candida covers large areas in a short time by frequent use of its furca and has a higher feeding rate than O.armatus. Colonies of slowly growing fungi are thus more likely to survive from the prudent grazing of O.armatus than from the intense grazing by F.candida.

d) Hanlon (1981) showed that high abundance of grazing Collembola in combination with low nutrient quality of the substrate had a dramatic effect in reducing the fungal growth and metabolism. In our experiments we used an abundance of O.armatus close to or slightly above the average density of Collembola found in a coniferous forest soil in S Scandinavia. Although O.armatus may be one of the most abundant species in the mor horizon, other species may add to the grazing pressure on a fungus and change its growth pattern. The effect of nutrient quality on fungal growth is difficult to evaluate separately from the influence of other parameters. In our respiration experiment we used a high quality substrate for the fungus and found a reduction in mycelial growth in the presence of metals. No evidence for reduced

growth was indicated in the metal polluted soil (Fig.2), presumably because the autoclaving made nutrients more available to the fungus. Nevertheless, grazed colonies on substrate containing metals had a higher respiration rate than ungrazed colonies on the same substrate, which would not be predicted from Hanlon's (1981) results, unless the nutrient quality of the fungus was unaffected by the metal uptake.

e) The growth of M.isabellina did not increase significantly in the metal polluted soil, while the respiration increased significantly from the fungus growing on metal contaminated agar. Since the respiration increase did not lead to a growth increase, the fungus presumably had to allocate more of its assimilated energy to detoxification. Grazing increased respiration rate in metal polluted Mortierella more than in the unpolluted, but a corresponding growth increase was not found. As a matter of fact, the metal polluted fungi showed a slight decrease in growth after three weeks incubation. We do not know whether the rapidly growing mycelium accumulated metals to such a level that growth was inhibited or whether the grazing intensity decreased because Onychiurus became inactive due to high concentrations of metals or avoided the toxic fungus (the same number of Collembola were found in polluted and unpolluted soil at the end of the experiment).

CONCLUSION

Our experiments clearly demonstrate that respiration and growth of M.isabellina was stimulated by grazing O.armatus. The exact mechanism for the stimulation remains unclear. It should be emphasized that the experiments do not exclude physical improvement of the substrate by animals as a factor stimulating fungal growth but they show that grazing cannot be rejected as a hypothesis for increased fungal activity.

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